Emergence of CTX-M-15 type extended-spectrum β-lactamase-producing Salmonella spp. in Kuwait and the United Arab Emirates

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INTRODUCTION

Salmonella spp. are an important cause of enteric fever and gastroenteritis in humans worldwide. The organisms are transmitted by contaminated food and inadequate hygiene. Typhoidal and serious invasive non-typhoidal Salmonella spp. infections are usually treated with antimicrobial agents. Fluoroquinolones and β-lactams are the drugs of choice for invasive salmonella infections. However, resistance has emerged to various classes of antibiotics in many parts of the world with the spread of resistant strains. Both healthcare-associated outbreaks and community outbreaks have been reported (Arlet et al., 2006).

In Salmonella, resistance to cefepime is largely due to production of extended-spectrum β-lactamases (ESBLs). Most ESBLs in Salmonella are derivatives of TEM and SHV β-lactamase families. Other groups, including PER and CTX-M types, have been described recently (Bonnet, 2004; Bradford, 2001). Also, β-lactamases belonging to either Ambler class B (metallo-β-lactamase) or class A, such as KPC-2, able to hydrolyse carbapenems, have been described (Miriagou et al., 2003).

CTX-M type ESBLs or cefotaximases, belong to class A β-lactamases and are encoded by blaCTX-M genes located in a plasmid or on the chromosome (Rodriguez et al., 2004). Different elements may be involved in the mobilization of blaCTX-M genes. Studies with plasmids have confirmed the potential involvement of the insertion sequence IS60 in the mobility of blaCTX-M (Cao et al., 2002). CTX-M enzymes comprise a rapidly growing family of enzymes disseminated in several parts of the world (Bonnet, 2004). A concern is the fact that CTX-M type ESBLs display a level of resistance to cefotaxime (Ctx) and ceftriaxone (Cro) significantly higher than that to ceftazidime (Caz) (Bradford, 2001), although Poirel et al. (2002) have described some CTX-Ms, including CTX-M-15, with good activity against Caz. Caz MICs for microorganisms producing CTX-M type ESBLs are usually within the susceptible range. Therefore, the use of Caz resistance as an indicator of ESBL production may miss ESBL-producing bacteria in the clinical microbiology laboratory. The aim of the study was to find out the burden of CTX-M-15-producing Salmonella spp. in Kuwait and United Arab Emirates (UAE), and the problems these strains pose for treatment.

Abbreviations: Amp, ampicillin; Caz, ceftazidime; Cro, ceftriaxone; Ctx, cefotaxime; Cxrm, cefuroxime; ESBL, extended-spectrum β-lactamase; UAE, United Arab Emirates.
METHODS

Selection of strains with β-lactam resistance phenotype. Salmonella spp. isolated from the stool samples of patients with acute diarrhoea were collected between January 2003 and March 2006 from Mubarak Al Kabir Hospital and Infectious Disease Hospital, Kuwait, and Tawam Hospital, Al Ain, UAE. Only single stool isolates for each patient were evaluated. A total of 407 isolates were collected and stored at −80°C until used. These comprised 284 isolates [247 non-typhoidal Salmonella, 25 Salmonella enterica serotype Typhi (S. Typhi) and 12 S. enterica serotype Paratyphi (S. Paratyphi) B and C] from Kuwait and 123 isolates (122 non-typhoidal Salmonella and 1 S. Typhi) from UAE. They were screened for phenotypes consistent with possible ESBL production using the breakpoint method described by Batchelor et al. (2005). Breakpoints used to identify resistance to the β-lactam antibiotics were as follows: ampicillin (Amp) (high-level resistance), >128 μg ml−1; cefuroxime (Cxm), >16 μg ml−1; Ctx, >1 μg ml−1; Cro, >1 μg ml−1; Caz, >1 μg ml−1. Determination of the full MICs for Ctx, Cro, Cxm and Caz for the blaCTX-M positive isolates (see below) was carried out using the Etest method (AB Biodisk). The results were collated according to the interpretative criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, 2005).

Detection of ESBL production. ESBL production by selected isolates was detected by the Etest ESBL method using both Caz/Caz combined with clavulanic acid and Ctx/Ctx combined with clavulanic acid strips (AB Biodisk). The tests were carried out and results interpreted as per the manufacturer’s instructions. In-house known ESBL-producing Escherichia coli and ESBL-negative strains were used as controls.

Detection of resistance genes. DNA template was obtained by using NucleoSpin tissue kit (Macherey-Nagel). ESBL-producing strains were analyzed by PCR to detect the presence of blaCTX-M, blasHV and blatem genes. PCR amplification was performed using published primer pairs (Cao et al., 2002), which are as follows: MA-1 5’-SCS ATG TGC AGY ACC AGT AA-3’ and MA-2 5’-CCG CRA TAT GRT TGG TGG TG-3’ (for blatem); OS-5 5’-TTA TCT CCC TGT TAG GCA CC-3’ and OS-6 5’-GAT TTG GTT CAT TCG CTC GG-3’ (for blatem), and C 5’-TGG GGG AAA TGT GCG CG-3’ and D 5’-TGC TTA ATC AGT GAG GCA CC-3’ (blasHV). PCR was carried out with the buffer supplied by the manufacturer using 1 μl each primer and 0.25 μl Taq polymerase [5 U (μl PE)] (GeneAmp Gold PCR reagent kit; Applied Biosystems). The reaction mixture (25 μl) was amplified using the following programs: 5 min 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C, with a final 10 min at 72°C (for blatem); 5 min 94°C, 30 cycles of 45 s at 94°C, 60 s at 55°C and 60 s at 72°C, with a final 5 min 72°C (for blatem); 30 cycles of 90 s at 94°C, 30 s at 60°C and 60 s at 72°C, with a final 10 min at 72°C (for blasHV). The amplicon sizes were 550, 797 and 971 bp for blatem, blasHV and blatem, respectively. The positive control for blatem and blasHV was Salmonella strain 971 and the negative control was Salmonella strain C600. The positive control for blasHV was Salmonella F1533 and the negative control was C600.

The strains with PCR amplicons positive for blatem were selected for sequencing. PCR products were purified using a NucleoSpin extract II kit (Macherey-Nagel) and sequenced using BigDye terminator cycle sequencing kit version 3.1 on ABI 3100 genetic analyser (Applied Biosystems). The nucleotide sequences were compared with the previously described sequences in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

Detection of ISEp1. The genetic environment of the blatem gene was determined initially by sequencing this short fragment by PCR. Primers, ISEp1A (5’-GCA GTT TTG TTT CTC C-3’) and ISEp1B (5’-ATT TCC GCA CGG TTT GC-3’) (Lartigue et al., 2004) were used. PCR was performed in a 25 μl mixture containing 1 μl sterile Milli Q distilled water, 2.5 μl 10X buffer PE, 2.5 μl MgCl2, 1 μl primer ISEp1A (5 μM), 1 μl primer ISEp1B (5 μM), 1 μl dNTPs (10 mM PE), 0.3 μl Taq polymerase [5 U (μl PE)] (GeneAmp Gold PCR reagent kit; Applied Biosystems). The PCR parameters included a denaturation step (5 min at 94°C), which was followed by 35 cycles of amplification (30 s of denaturation at 94°C, 30 s of annealing at 57°C, 60 s of elongation at 72°C) and 7 min at 72°C final extension. The PCR products were analysed by electrophoresis in a 1% agarose gel. Salmonella strain 971 and Salmonella strain C600 were used as positive and negative controls, respectively.

Molecular typing. Typing of CTX-M-15 positive strains was performed using PFGE, with XhoI-digested DNA separated by electrophoresis in a 1.2% agarose gel as described by the CDC (2002). The strains were compared based on differences in the number and mobility of bands.

RESULTS

Selected bacterial isolates

Based on high-level resistance to Amp and resistance to at least one of the cephalosporins, a total of 116 (28.5%) out of the 407 isolates were further studied. These included 79 strains from Kuwait and 37 from UAE. These isolates were made up of 77 non-typhoidal Salmonella spp., 2 S. Typhi from Kuwait, and 36 non-typhoidal Salmonella spp. and 1 S. Typhi from UAE.

Prevalence of ESBL-producing Salmonella isolates

As can be seen in ESBL distribution shown in Table 1, of the total of 116 selected isolates, 69 (59.5%) were ESBL-producers; 50 (72.5%) and 19 (27.5%) of these were from Kuwait and UAE, respectively. Of the 50 ESBL-positive isolates in Kuwait, 72% belonged to S. enterica serogroups B and C. In contrast, 63.1% ESBL-positive isolates in the UAE belonged to serogroups B and C.

Characterization of the resistance genes

All 116 selected isolates were screened by PCR assays for blatem, blasHV and blatem genes. Sequencing, followed by BLAST searches, confirmed blatem as blatem-15. Of these 116 isolates, 29 (25%) carried the blatem gene (21 from Kuwait and 8 from UAE). The blatem-15 gene was found in 14 (12.1%) isolates, i.e. 12.1% of total resistant isolates and 20.3% of the ESBL-positive isolates (13 from Kuwait and 1 from UAE), 9 of which also harboured the blatem gene. However, it is noteworthy that the blasHV gene was not detected in any of the Salmonella isolates. Of the 14 isolates positive for the blatem-15 gene, 2 (14.3%) were S. Typhi and 10 (71.4%) of these isolates also carried the blatem gene. As shown in Table 2, within the blatem-15 positive group, S. enterica serotype Typhimurium (S. Typhimurium) were the most resistant, followed by Salmonella serogroup C and Salmonella serogroup B. The two S. Typhi did not carry the blasHV gene and...
were just as resistant to cefotaxime. The MICs of Caz for all these isolates ranged from 1 μg to 16 μg ml⁻¹ with the two S. Typhi being the most resistant.

All the 14 patients infected with blaCTX-M-15 gene-carrying strains were non-Kuwaitis, 10 (71.4 %) of whom were non-Kuwaiti Arab nationals from other Gulf states and the Lebanon. The remaining four patients comprised three Indians and one Pakistani.

**Molecular typing**

Fig. 1 shows the XbaI PFGE fingerprints for the 13 Kuwaiti isolates. Five of the thirteen blaCTX-M-15 positive isolates from Kuwait formed a cluster showing identical patterns (see lanes 1, 2, 4, 7 and 9) while the rest presented with different PFGE restriction profiles. The single positive isolate from UAE (U38) had a different restriction profile from the Kuwaiti strains (not shown). As shown in Fig. 1, the S. Typhi isolates (K181, K238) had different restriction patterns.

**DISCUSSION**

In this study, CTX-M and TEM ESBL were the main enzymes found in our isolates. None of the clinical isolates produced SHV ESBL. β-Lactamases are the predominant

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Table 1. Prevalence of ESBL and CTX-M types in *Salmonella* spp. isolated from Kuwait and the UAE

<table>
<thead>
<tr>
<th><em>Salmonella</em> spp. (no. from Kuwait/no. from the UAE)</th>
<th>No. (%) of ESBL- &amp; CTX-M-positive strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kuwait</td>
</tr>
<tr>
<td></td>
<td>ESBL</td>
</tr>
<tr>
<td>S. Typhimurium (19/0)</td>
<td>5 (10)</td>
</tr>
<tr>
<td>S. Enteritidis (23/0)</td>
<td>2 (4 )</td>
</tr>
<tr>
<td><em>Salmonella</em> group B (60/24)</td>
<td>17 (34)</td>
</tr>
<tr>
<td><em>Salmonella</em> group C (76/30)</td>
<td>19 (38)</td>
</tr>
<tr>
<td><em>Salmonella</em> group D (35/39)</td>
<td>3 (6 )</td>
</tr>
<tr>
<td>S. Paratyphi (12/0)</td>
<td>1 (2 )</td>
</tr>
<tr>
<td>S. Typhi (25/1)</td>
<td>2 (4 )</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.* (21/0)</td>
<td>0</td>
</tr>
<tr>
<td>Ungroupable (13/30)</td>
<td>1 (2 )</td>
</tr>
<tr>
<td>Total (284/123)</td>
<td>50 (17.6)</td>
</tr>
</tbody>
</table>

*Salmonella* spp. = *Salmonella* serogroup E (8), *Salmonella* serogroup G (10), *Salmonella* serogroup H (3).

Table 2. Characteristics of blaCTX-M-producing *Salmonella* isolates from Kuwait and the UAE

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Serotype/serogroup</th>
<th>Date isolated</th>
<th>MIC of:</th>
<th>Type of ESBL</th>
<th>IS*EcpI</th>
<th>Patient nationality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Amp</td>
<td>Ctx</td>
<td>Caz</td>
<td>Cxm*</td>
</tr>
<tr>
<td>K16</td>
<td>S. Typhimurium</td>
<td>17.06.03</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>2</td>
<td>&gt;256</td>
</tr>
<tr>
<td>K147</td>
<td>S. Typhimurium</td>
<td>11.05.04</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>3</td>
<td>&gt;256</td>
</tr>
<tr>
<td>K152</td>
<td>S. Typhimurium</td>
<td>18.05.04</td>
<td>&gt;256</td>
<td>64</td>
<td>1</td>
<td>128</td>
</tr>
<tr>
<td>K180</td>
<td>S. Enteritidis</td>
<td>28.06.04</td>
<td>&gt;256</td>
<td>32</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>K181</td>
<td>S. Typhi</td>
<td>28.06.04</td>
<td>&gt;256</td>
<td>16</td>
<td>12</td>
<td>64</td>
</tr>
<tr>
<td>K205</td>
<td><em>Salmonella</em> spp.</td>
<td>02.08.04</td>
<td>&gt;256</td>
<td>16</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>K235</td>
<td><em>Salmonella</em> B</td>
<td>09.10.04</td>
<td>&gt;256</td>
<td>32</td>
<td>1.5</td>
<td>128</td>
</tr>
<tr>
<td>K236</td>
<td><em>Salmonella</em> B</td>
<td>11.10.04</td>
<td>&gt;256</td>
<td>16</td>
<td>0.25</td>
<td>128</td>
</tr>
<tr>
<td>K237</td>
<td><em>Salmonella</em> B</td>
<td>12.10.04</td>
<td>&gt;256</td>
<td>16</td>
<td>2</td>
<td>&gt;256</td>
</tr>
<tr>
<td>K238</td>
<td>S. Typhi</td>
<td>12.10.04</td>
<td>&gt;256</td>
<td>32</td>
<td>16</td>
<td>&gt;256</td>
</tr>
<tr>
<td>K247</td>
<td><em>Salmonella</em> C2</td>
<td>02.11.04</td>
<td>&gt;256</td>
<td>32</td>
<td>1.5</td>
<td>&gt;256</td>
</tr>
<tr>
<td>K270</td>
<td>S. Typhimurium</td>
<td>09.05.05</td>
<td>&gt;256</td>
<td>64</td>
<td>12</td>
<td>&gt;256</td>
</tr>
<tr>
<td>K286</td>
<td><em>Salmonella</em> B</td>
<td>13.06.05</td>
<td>&gt;256</td>
<td>32</td>
<td>4</td>
<td>128</td>
</tr>
<tr>
<td>U38</td>
<td><em>Salmonella</em> C</td>
<td>24.11.02</td>
<td>&gt;256</td>
<td>128</td>
<td>2</td>
<td>128</td>
</tr>
</tbody>
</table>

NK, Non-Kuwaiti.
cause of resistance to β-lactam antibiotics in Gram-negative bacteria. Among the Ambler class A ESBL, the TEM, SHV and CTX-M enzymes are the most widely distributed. CTX-M enzymes emerged in the late 1980s, shortly after the introduction of Ctx in clinical practice. Since then, wide dissemination of strains carrying these enzymes has occurred, and led to several outbreaks in hospitals and communities (Arlet et al., 2006). In 2004, Salmonella resistant to extended-spectrum cephalosporins were identified in 43 countries encompassing Europe, Latin America, the USA, Taiwan and the Western Pacific (Winokur et al., 2001; Dunne et al., 2000; Li et al., 2005; Su et al., 2005), and its prevalence ranged between 0 and 3.4 %.

This is believed to be the first report of CTX-M type ESBL-producing Salmonella in Kuwait and the UAE, and one of the very few reports on the ESBL-producing Salmonella in the Middle East (Hammami et al., 1991; Moubareck et al., 2005). The prevalence of ESBL-producing Salmonella was about 17 % with a higher proportion (73 %) of these attributable to isolates from Kuwait. This prevalence is much higher than any reported prevalence rates in Salmonella spp., such as the prevalence of 1.5 % reported in the Taiwan (Li et al., 2005; Su et al., 2005), 1.9 % in the USA (Dunne et al., 2000), 2.4 % in Latin America (Winokur et al., 2001), 0.8 % in Europe and 3.4 % in the Western Pacific region (Winokur et al., 2001). The reason for the high prevalence of ESBL-producing Salmonella in our study may be related to the level of usage of the drug in the community, although we do not have any quantitative data on this.

Our study reports for what is believed to be the first time, the explosive spread of CTX-M-15-producing Salmonella isolates and the presence of bla_{CTX-M} in S. Typhi in the Gulf region, particularly from Kuwait. Until recently, only CTX-M-1, CTX-M-2 and CTX-M-9 have been described in Salmonella spp. (Miriagou et al., 2004). There have been reports of outbreaks due to CTX-M-2 ESBL-producing Salmonella in Argentina (Rossi et al., 1995; Bauernfeind et al., 1996; Orman et al., 2002) and CTX-M-4- or CTX-M-5-producing S. Typhimurium in Russia, Belarus, Hungary and Latvia between 1996 and 1999 (Edelstein et al., 2004; Tassios et al., 1999; Bradford et al., 1998). Outbreaks due to CTX-M-15-producing Salmonella strains have not been reported previously. However, sporadic individual infections involving CTX-M-15 ESBL-producing Salmonella spp. have been reported in Lebanon and elsewhere (Batchelor et al., 2005; Moubareck et al., 2005; Morris et al., 2006; Kim et al., 2007).

In the past, CTX-M-15 ESBL enzyme has been found almost exclusively in E. coli and Klebsiella spp., and has been a cause of concern for many clinical microbiologists and infectious diseases experts in the UK and other European countries (Livermore & Hawkey, 2005); it is associated with multiple drug resistance and is spreading rapidly. We have found a sudden and phenomenal increase in the prevalence of E. coli and Klebsiella clinical isolates producing this enzyme (Poirel et al., 2007). This parallels a sharp rise in the isolation of E. coli and Klebsiella resistant to Ctx/Cro and many other antibiotics in our teaching hospitals. In a previous study in 1998 (Jamal et al., 1998) on 661 strains of Salmonella spp., only 0.3 % of the strains were resistant to Ctx in Kuwait. At present, the resistance rate has gone up fivefold (personal observation). It is conceivable that the Salmonella strains in Kuwait and UAE could have acquired the gene encoding this enzyme from E. coli and Klebsiella in the community, and then it spread among the various species and serotypes of Salmonella. This speculation is supported by the fact that the mobile genetic element ISEcpI, a single copy insertion sequence responsible for mobilization of bla genes and identified upstream of several bla_{CTX-M} genes (Walther-Rasmussen & Høiby, 2004), was found in association with about 72 % of our isolates. The only other ESBL found in Salmonella in the Middle East is SHV-2 type, which was first reported during a nosocomial outbreak of acute gastroenteritis due to ‘Salmonella wien’ in a neonatal intensive care unit in Tunisia (Hammami et al., 1991).

It has been reported from Europe that bla_{CTX-M} determinants are often linked to infections in travellers returning to their countries (Batchelor et al., 2005). In 2005, Moubareck and colleagues reported for the first time an ESBL-producing S. Typhimurium of CTX-M-15-type in the Lebanon (Moubareck et al., 2005). It was isolated from the stool sample of a 6-year-old boy who did not have a history of travel outside of the Lebanon. Similarly, about 72 % of our CTX-M-15 ESBL-producing isolates were from patients of non-Kuwaiti Arab origin who had no history of

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**Fig. 1.** PFGE pattern of the 13 CTX-M-15 ESBL-positive Salmonella spp. isolated in Kuwait and the ladder size markers. Lanes: 1, Salmonella group B (K238); 2, S. Typhimurium (K16); 3, S. Typhimurium (K147); 4, S. Typhimurium (K152); 5, S. Typhi (K181); 6, S. Typhi (238); 7, S. Typhimurium (K270); 8, Salmonella group B (K235); 9, Salmonella group B (K237); 10, S. enterica serotype Enteritidis (S. Enteritidis) (K180), 11, Salmonella group B (286), 12, Salmonella spp. (K205), 13, Salmonella group C2 (K247); 14, Pulse marker 50–1000 kb. Strains in lanes 1, 2, 4, 7 and 9 demonstrated PFGE clonal relatedness.
travel in the 6 months preceding their infections. Three of the remaining four patients were of Indian origin and also did not have a history of recent travels. Four of five CTX-M-15 ESBL-producing strains with PFGE clonal relatedness (94% similarity) were S. Typhimurium isolated from unrelated non-Kuwait Arabs, who did not travel out of Kuwait prior to infection, and that were isolated at different periods during the 2 year study. This probably indicates a clonal spread of resistance in this species. The remaining 10 isolates were of diverse origin, suggesting that the spread of the CTX-M-15-mediated resistance may be due to horizontal transfer rather than to spread of a single clone. Although no transfer experiments or plasmid analyses were performed on our isolates, it is reasonable to speculate that these genes are transferable as has been amply demonstrated in the literature (Batchelor et al., 2005; Jin & Ling, 2006).

We did not find any \( \text{bla}_{\text{SHV}} \) genes in our isolates. As the members of \( \text{bla}_{\text{SHV}} \) group are extremely diverse, we have only included primers able to detect \( \text{bla}_{\text{SHV}} \) genes that have been found previously in Salmonella. Therefore, we cannot exclude that some of our isolates may contain \( \text{bla}_{\text{SHV}} \) genes not previously described in these species.

Many expatriate workers reside in Kuwait and the UAE, and there is a constant movement of people to/from different parts of the world. Usually, we see importation of many infectious diseases from Asia and Africa into the Middle East. However, CTX-M-15-positive Salmonella were mostly detected among the Arab population in our study, with the possibility of local origin or origin from other Arab countries. The possibility exists that these Salmonella strains could be carried to Asia and Africa, initiating a reverse migration of resistant strains. Already, we have detected the strains in some individuals from the Indian subcontinent.

In conclusion, the finding of the \( \text{bla}_{\text{CTX-M-15}} \) gene in Salmonella in association with \( \text{IS}_{\text{Ecp1}} \) is a serious concern, because of the propensity of the latter to facilitate the spread of resistance. This finding is worrying as the treatment of infections involving multiply resistant strains is often difficult. Therefore, measures such as improved food and personal hygiene, and organizing data from patients with similar problems into the same statistical group (patient cohorting), could help to prevent further spread of resistance strains.

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