Characterization of AmpC-Mediated Resistance in Clinical Salmonella Isolates Recovered from Humans during the Period 1992 to 2003 in England and Wales

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Received 28 October 2004/Returned for modification 31 December 2004/Accepted 23 January 2005

The increase in AmpC-mediated resistance in salmonellae constitutes a serious public health concern, since these enzymes confer resistance to a wide range of β-lactams. One hundred six isolates were selected from 278,308 Salmonella isolates based on resistance to ampicillin and cephalosporins and were subjected to further characterization. Nine isolates had a cefoxitin inhibition diameter ≤17 mm and were proven to be AmpC positive by multiplex PCR. Sequence analysis revealed the presence of bla_DHA-1, bla_CMY-2, and bla_CMY-4 genes. All nine isolates presented different pulsed-field gel electrophoresis restriction profiles. The AmpC genetic determinants were present in transferable plasmids of around 11, 42, 70, 98, and 99 Mda. A combination of size and restriction fragment length polymorphism (RFLP) analysis showed that all the bla_CMY plasmids investigated in our study were different, which suggests that bla_CMY may be located in different plasmid environments. Some United Kingdom isolates linked to foreign travel showed RFLP plasmid patterns consistent with plasmids previously seen in the United States, which suggests that bla_CMY has also been disseminated through plasmid transfer. The fact that two of the domestically acquired United Kingdom isolates presented previously unseen RFLP plasmid patterns could indicate that these strains have followed routes different from those prevalent in North America or other parts of the world. This study represents the first report of bla_CMY genes in Salmonella isolates in the United Kingdom and the first report of CMY-4 in Salmonella enterica serotype Senftenberg worldwide.

Plasmid-mediated AmpC β-lactamases originate from the transfer of chromosomal genes onto plasmids. This transfer has resulted in plasmid-mediated AmpC enzymes in isolates of Escherichia coli, Klebsiella pneumoniae, Salmonella spp., Citrobacter freundii, Enterobacter aerogenes, and Proteus mirabilis. These plasmid-mediated β-lactamases have similar substrate profiles to the parental enzymes from which they appear to be derived, although the vast majority are noninducible. Several AmpC-type enzymes have been described including FOX, MOX, CMY, DHA, ACC, MIR, ACT, and LAT. Of particular interest in Salmonella are the CMY enzymes, especially CMY-2. There are currently 13 bla_CMY genes identified, falling into two distinct groups based on DNA sequence homology. One group includes the genes CMY-2 to -7, LAT-1 to -4, and BIL-1 and originates from C. freundii (21), while the second group includes the genes for MOX-1 and -2, CMY-1, and CMY-8 to -11 and has an unknown origin (21). CMY-2 was first identified in a human clinical case of K. pneumoniae from Greece (4). Since then, CMY-2 has been identified in a large number of different human and animal Salmonella serovars (Newport [22], Typhimurium [1], Hadar [25], Senftenberg [12], Infantis [7, 14], Agona [7], Ohio [1], Bredeney [1], Wien [2], Heidelberg [16], Mikawasima [17], Montevideo [17], Oranienburg [19], and Choleraesuis [8]) from humans and animals.

Most Salmonella infections result in moderate gastroenteritis, which resolves spontaneously. However, systemic infections, such as bacteremia and meningitis, may also develop and require antibiotic treatment. These invasive infections commonly occur in immune-compromised patients, the elderly, children, and infants. Antimicrobial agents are also commonly used for the empirical treatment of patients with severe diarrhea. Fluoroquinolones are often used for treating adults with complicated salmonella infections but are not approved for use in children. This patient group is commonly treated with expanded-spectrum cephalosporins because of their favorable pharmacokinetic properties. AmpC enzymes confer resistance to all β-lactams except for amdinocillin, cefepime, cepacrime, and carbapenems. The increase of AmpC-mediated resistance in salmonellae seriously compromises the use of these drugs and constitutes a serious public health concern.

In order to assess the current presence of organisms/genes of concern in England and Wales, the present work aimed to screen a large collection of salmonella isolates of human origin with regard to the presence of AmpC enzymes. Subsequently the genes and gene-bearing plasmids of the positive strains were characterized in detail. The information presented is of
special relevance to understanding the epidemiology of β-lactam resistance in *Salmonella* in the United Kingdom.

### MATERIALS AND METHODS

Isolates and determination of β-lactam resistance pheno- and genotypes. Isolates from the *Salmonella* strain collection at the Laboratory for Enteric Pathogens, Health Protection Agency (HPA), Colindale, United Kingdom, from the period 1992 to 2003 (total number, 278,308, comprising 31,339 from 1992, 29,276 from 1993, 29,514 from 1994, 28,588 from 1995, 28,210 from 1996, 31,480 from 1997, 23,161 from 1998, 16,957 from 1999, 14,465 from 2000, 16,038 from 2001, 14,427 from 2002, and 14,853 from 2003) were scrutinized for phenotypes consistent with possible ESBL production. These represent all human clinical and food *Salmonella* isolates submitted to the reference laboratory for England and Wales. Resistance to antimicrobials was determined using a breakpoint method in *Eco96ar* agar (9). The breakpoints (in mg/liter) for resistance to the β-lactams were as follows: ampicillin (high-level resistance) (A), 128; cephalaxin (Cx), 16; cefadroxil (Cr), 16; cefuroxime (Cf), 16; ceftriaxone (Cn), 1; ceftaxime (Ct), 1. Breakpoints for other antibiotics were as described previously (24).

Selected isolates were analyzed by an AmpC multiplex PCR (15). Subsequently, for those showing positive results, the full-length bla*

**-lactam resistance phenotypes and genotypes.** Conjugations were performed with the bla*

**CMY**-positive *Salmonella* isolates and a rifampin-resistant recipient, *E. coli* K-12 208764, using previously described methods (15). For the strains where transfer was not achieved by conjugation, transformation experiments were conducted as described before (14).

Restriction fragment length polymorphism (RFLP) for plasmid characterization. Transferable plasmids were purified from either transconjugant or transformant cells using a QIAGEN high-speed MIDI kit. Purified plasmid DNA (2 µg) was digested for 2 h at 37°C with 15 U of PstI (Promega), and the resulting products were separated by electrophoresis on 0.8% agarose gels at 45 V for 20 h with recirculation of the buffer (1 × Tris-acetate-EDTA) at 14°C. Digoxigenin-labeled DNA molecular weight marker II (Roche Molecular Biochemicals) was used as a size standard. Fractionated DNA was transferred to positively charged nylon membranes (Roche Molecular Biochemicals) using a vacuum blotting apparatus (Pharmacia Biotech, Herts, United Kingdom) connected to a variable pump set at 40 mbar for 1 h. Membranes were rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and air dried before DNA was fixed to the membranes by cross-linking under UV light. Membranes were prehybridized for 4 h at 42°C in 20 ml of DIG Easy Hyb (Roche Molecular Biochemicals). A bla*

**-specific probe was generated by PCR with primers CITM and CITMR (21) and labeled with digoxigenin-11-dUTP using the DIG-High Prime labeling kit (Roche Molecular Biochemicals) and the chemiluminescent substrate CSPD as recommended by the supplier.

### RESULTS

#### Isolate selection process.

The criterion for selection of isolates was resistance to ampicillin plus at least one of the cephalosporins in our panel. One hundred six isolates were selected from the total 278,308 isolates analyzed based on this criterion and were subsequently subjected to further characterization. These isolates were screened further for susceptibility to amoxicillin-clavulanic acid, aztreonam, imipenem, and cefoxitin (Table 1) by a disk diffusion method (18). Nine isolates with a cefotaxin inhibition diameter ≤17 mm were selected as possible AmpC producers and characterized genetically.

#### Table 1. Characterization of AmpC-producing *Salmonella* isolates in England and Wales

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Serotype</th>
<th>Resistance pattern(^a)</th>
<th>Date isolated (mo-yr)</th>
<th>Size (MDa)</th>
<th>Transferable plasmid</th>
<th>AmpC gene</th>
<th>Amplification temperature (°C)</th>
<th>Conjugation</th>
<th>Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Senftenberg</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>1-1996</td>
<td>6</td>
<td>No</td>
<td>Amc</td>
<td>60</td>
<td>98</td>
<td>Conjugation</td>
</tr>
<tr>
<td>A3</td>
<td>Staffsberg</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>2-1999</td>
<td>17</td>
<td>6</td>
<td>DHA-1</td>
<td>60</td>
<td>98</td>
<td>Conjugation</td>
</tr>
<tr>
<td>A4</td>
<td>St Albans</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>6-1999</td>
<td>6</td>
<td>10</td>
<td>DHA-1</td>
<td>60</td>
<td>99</td>
<td>Transformation</td>
</tr>
<tr>
<td>A5</td>
<td>Aichi</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>9-2001</td>
<td>6</td>
<td>10</td>
<td>DHA-1</td>
<td>60</td>
<td>91</td>
<td>Transformation</td>
</tr>
<tr>
<td>A6</td>
<td>Senftenberg</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>12-2001</td>
<td>10</td>
<td>19</td>
<td>DHA-1</td>
<td>60</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>A7</td>
<td>Heidelberg</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>12-2001</td>
<td>12</td>
<td>19</td>
<td>DHA-1</td>
<td>60</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>A8</td>
<td>Halleberg</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>12-2001</td>
<td>10</td>
<td>19</td>
<td>DHA-1</td>
<td>60</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>A9</td>
<td>Typhimurium</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>9-2002</td>
<td>11</td>
<td>18</td>
<td>DHA-1</td>
<td>60</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>A10</td>
<td>Athens</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>8-2003</td>
<td>12</td>
<td>17</td>
<td>DHA-1</td>
<td>60</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>A11</td>
<td>Worthington</td>
<td>Chl Gen Kan Str Su Tet Trip Nal</td>
<td>7-2003</td>
<td>10</td>
<td>17</td>
<td>DHA-1</td>
<td>60</td>
<td>42</td>
<td>70</td>
</tr>
</tbody>
</table>

\(^a\) Amoxicillin (Amp), cephalexin (Lex), cephradine (Rad), cefuroxime (Cxm), ceftriaxone (Cro), cefotaxime (Ctx), chloramphenicol (Chl), gentamicin (Gen), neomycin (Ne), kanamycin (Kan), streptomycin (St), sulfonamide (Su), spectinomycin (Spt), tetracycline (Tet), trimethoprim (Tmp), furazolidone (Fu), nalidixic acid (Nal), cephalaxin (Cep), cefoxitin (Cfx). Antimicrobials in bold font are those where resistance was cotransferred to a recipient with the AmpC plasmid.
Resistance characterization and molecular fingerprinting. The nine isolates selected were proven to be AmpC positive by the multiplex PCR. Two of the strains were PCR positive for the DHA group, while the rest produced a product consistent with the CIT group (comprises LAT-1 to -4, CMY-2 to -7, and BIL-1). Sequencing followed by BLAST searches confirmed the identity of the different genes, and this information is summarized in Table 1. All the isolates investigated presented different PFGE restriction profiles (Fig. 1).

Transferability of resistance. Transconjugants were obtained only for four of the strains; for the other five strains, *E. coli* transformants were obtained after electroporation (Table 1).

Isolates A1 and A3 were the subject of a recent communication (13). Isolate A6 transferred a plasmid of approximately 42 MDa. Isolates A4, A5, and A11 transferred a plasmid of around 70 MDa. Isolates A7 and A10 transferred a plasmid of around 98 MDa. Finally, isolate A9 transferred a plasmid of around 11 MDa. PCR analysis of the transconjugants and transformants showed that the genetic determinants were present in the specific plasmids acquired by the recipient strains. In addition, the transconjugant/transformant cells acquired resistance to the same range of β-lactams that was present in the donor strains. For those strains where resistance to antimicrobials other than β-lactams was seen (A1, A3, A6, A7, A9, A10, A11), sensitivity testing of the resulting transconjugant/transformant cells showed that some of these resistances were cotransferred with the AmpC plasmids (Table 1).

RFLP testing showed that the *bla*<sub>CMY-2</sub> probe hybridized to bands of approximately 12 and 0.8 kb in the analysis of plasmids from strains A7 and A11, to bands of approximately 12, 5, and 0.8 kb in analysis of the plasmid from A9, to bands of 13 kb and 0.8 kb in analysis of the plasmid from A10, to bands of 4, 3, 2.5, and 0.8 kb in analysis of the plasmid from A4, to bands of 4.5 kb and 0.8 kb in analysis of the plasmid from A5, and finally to bands of 1,000 and 800 bp in the analysis of the plasmid from A6 (Fig. 2).

**DISCUSSION**

The first reports of AmpC enzymes from humans in the United Kingdom were of *bla*<sub>BIL-1</sub> in *E. coli* in 1992 and 1997 (11, 20), *bla*<sub>CMY-3</sub> in *K. pneumoniae* in 1995, and *bla*<sub>CMY-4</sub> in *E. coli* in 1999 (23). Recently, we have reported the isolation of a *bla*<sub>CMY-2</sub>-positive *Salmonella* serovar Bredeney isolate from an avian source (15) and a *bla*<sub>CMY-2</sub>-positive *E. coli* isolate from cattle (3). The present study represents the first report of *bla*<sub>CMY</sub> genes in *Salmonella* isolates in the United Kingdom and of CMY-4 in *Salmonella* serovar Senftenberg worldwide.
This enzyme was found for the first time in *Salmonella* in France (2) in 2003, and no further reports exist to date. There is the suggestion that the critical mass for extended-spectrum cephalosporins may have been exceeded due to the intensive use of these drugs in clinical practice over the last decades. There must be an explanation of why the current situation is so different in different countries. As an example, in the United States, where decreased susceptibility to ceftriaxone is mediated almost exclusively through production of CMY-2 (10), the 2001 National Antimicrobial Resistance Monitoring System (NARMS) data showed that 49 non-serotype Typhi *Salmonella* isolates (3%) had decreased susceptibility to ceftriaxone, of which 34 (71%) were resistant to ceftriaxone. In contrast, very little resistance to extended-spectrum cephalosporins has been seen in salmonellae in England and Wales in the last 10 years, with only 7 out of 278,308 isolates found to carry *bla*<sub>CMY</sub>-2. It is difficult to determine whether the level of cephalosporin use may be the key factor for these differences. The reason is the inability to determine the level of use for each drug class, because there is no transparency in the sales data of the different drugs. In addition, our ability to interpret this information is complicated by the fact that in some cases other antimicrobial agents may be applying selective pressure. It has been shown that many of these plasmids encode resistance genes for different antibiotic classes.

A combination of size and RFLP analysis showed that all the plasmids investigated in our study were different, which suggests that *bla*<sub>CMY</sub>-2 may be located in different plasmid environments. However, RFLP patterns from plasmids A7 and A11 showed hybridizing-fragment sizes compatible with those reported for the type A plasmids described by Giles et al. (10), although a direct comparison would be necessary to fully support this observation. A7 and A11 correspond to isolates from serum types Heidelberg and Worthington, acquired following visits to Greece and Iraq, respectively. Isolate A10 (*Salmonella* serotype Anatum, acquired following a visit to the United States) showed an RFLP pattern that could also have been confused with type A due to the shorter electrophoresis times used in the study of Giles et al. Isolate A9 (*Salmonella* serotype Typhimurium, acquired following a visit to Mexico) showed an RFLP profile that could be related to that of type A plasmids apart from the presence of an extra band of 5 kb. Isolate A4 (*Salmonella* serotype [4,5,12:I–]), acquired following a visit to Gambia) showed an RFLP profile that could be related to that of type B plasmids apart from the presence of an extra band of 4.3 kb. All these infections were linked to foreign travel and showed RFLP plasmid patterns consistent with those of plasmids previously seen in the United States. This adds strength to the suggestion that *bla*<sub>CMY</sub>-2 has been disseminated primarily through plasmid transfer (10). The fact that two of the isolates that appear to have been acquired domestically in the United Kingdom (A5, A6) presented previously unseen RFLP plasmid patterns suggests that mobilization of the gene to multiple plasmid backbones may also be a possible mechanism. It could be hypothesized that strains developed in the United Kingdom have followed different routes from those prevalent in North America or other parts of the world. A harmonized way of studying plasmids from different countries is needed to ascertain the relative importance of both types of dissemination in the epidemiology of *bla*<sub>CMY</sub>-2-mediated resistance.

Conjugal transfer of type A plasmids has been reported to be at a very low frequency (10). In agreement with this observation, in our study none of the type A-related plasmids were transferable by conjugation. In contrast, conjugal transfer was successful for type B-related plasmids (A4) and the newly found types (A10, A5, A6).

Plasmids of type A confer a multidrug-resistant phenotype not only to β-lactams but also to multiple classes of antimicrobials (10). In our study all isolates with type A-related plasmids were also resistant to a variety of antimicrobials (among them, chloramphenicol, streptomycin, sulfonamide, and tetracycline). This once again shows that coselection of these plasmids by the use of other antimicrobial classes is possible. Type B plasmids encode resistance only to β-lactam antibiotics (5); this was also the case for the plasmid from isolate A4, which was resistant only to this group of drugs. In addition, the plasmid from isolate A5 encoded only resistance to β-lactams. Four of the nine isolates (A1, A2, A6, A11) presented a ciprofloxacin MIC ≥32 mg/liter, and three of these infections appeared to be domestically acquired. This represents a serious cause for concern due to the fact that the two classes of drugs of choice for complicated cases of salmonellosis could have been inadequate for therapy.

We have found that AmpC-mediated resistance in *Salmonella* isolates in the United Kingdom remains rare, and in most cases the isolates originate from foreign-travel-acquired infections. We have seen also some domestic cases, and we must therefore remain vigilant for the development of resistance to these important drugs, especially in the case of important zoonotic organisms such as salmonellae.

ACKNOWLEDGMENT

This work was funded by the Department for Environment, Food and Rural Affairs, United Kingdom, project VM02136.

REFERENCES


