Increasing ceftriaxone resistance in *Salmonella* isolates from a university hospital in Taiwan

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Objectives: *Salmonella* infection is a distressing health problem worldwide. This study reports the changing epidemiology of *Salmonella* infections in Taiwan during 1999–2003, with emphasis on increasing ceftriaxone resistance.

Methods: Records of *Salmonella* clinical isolates in Chang Gung Memorial Hospital during 1999–2003 were reviewed. All isolates were identified and antimicrobial susceptibility determined by standard methods. A total of 22 ceftriaxone-resistant isolates were investigated by PCR sequencing of the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub> and *ampC* genes. Southern-blot hybridization was used to localize the *ampC* gene. Infrequent-restriction-site PCR was used to genotype these isolates.

Results: A total of 3635 *Salmonella* isolates, including 3592 (98.8%) non-typhoid *Salmonella*, were identified. Serogroup B (55.6%) remained the most predominant, but the prevalence has been decreasing. In contrast, serogroup D infections have increased significantly from 13.6 to 22.8%. Overall resistance to ampicillin and chloramphenicol remained high, with the highest rate (91% to both drugs) observed in *Salmonella enterica* serotype Choleraesuis in 2003. A sudden upsurge of ciprofloxacin resistance from zero to 69% was found in *S*. Choleraesuis. Ceftriaxone resistance increased in several serogroups (0.8–2.1%; average, 1.5%). The resistance was associated with plasmid-mediated *bla*<sub>CMY-2</sub> in 14 cases and extended-spectrum β-lactamases (ESBLs), including CTX-M-3 (*n* = 6), SHV-2a (*n* = 1) and SHV-12 (*n* = 1), in others. Diverse serotypes and genotypes were found among the ceftriaxone-resistant isolates.

Conclusions: Increasing ceftriaxone resistance in non-typhoid *Salmonella* appears to link to the spread of plasmid-mediated *ampC* or ESBL genes. Effective measures should be taken to prevent the problem worsening.

Keywords: antimicrobial resistance, *ampC*, extended-spectrum β-lactamases

**Introduction**

*Salmonella* infection remains a significant public health problem worldwide. In many countries, the prevalence of human infections caused by antimicrobial-resistant *Salmonella* has been on the rise.¹ Conventional antimicrobial agents, such as ampicillin, chloramphenicol and sulfamethoxazole/trimethoprim, were the drugs of choice in the treatment of salmonellosis before the 1980s. During the past two decades, *Salmonella* with multiple resistance to these drugs has been reported from many countries.²–⁴ Extended-spectrum cephalosporins and fluoroquinolones are recommended as alternatives in such a setting.²–⁵ However, since 1991, outbreaks or cases of infections caused by *Salmonella* resistant to extended-spectrum cephalosporins have
been increasingly reported.\textsuperscript{8–12} Growing resistance to fluoroquinolones was also noted.\textsuperscript{13}

Previous reports from Taiwan have indicated that antimicrobial resistance among clinical \textit{Salmonella} isolates is a serious problem.\textsuperscript{3,14–16} We conducted the present study to analyse the epidemiology of recent clinical isolates of \textit{Salmonella}, with a special focus on the increase in ceftriaxone resistance.

Materials and methods

Laboratory-based surveillance

Records of \textit{Salmonella} clinical isolates from the Department of Clinical Pathology in Chang Gung Memorial Hospital (CGMH) during 1999–2003 were reviewed. Because the policy of the requisition of bacterial cultures has remained the same throughout the years, any change in the proportion of isolates was presumed to be related to the frequency of infections. The CGMH is a 4000-bed university-affiliated medical centre located in northern Taiwan, but the patients are from almost anywhere in Taiwan, including the main and scattered islands.

Microbiological examination

All isolates were cultured and identified by standard methods.\textsuperscript{17} Serogroups of the \textit{Salmonella} isolates were determined by O antisera (Difco Laboratories, Detroit, MI, USA) using the slide agglutination method. Serotypes of ceftriaxone-resistant isolates were determined by H antisera (Difco Laboratories) using the tube agglutination method. \textit{Salmonella enterica} serotype Choleraesuis, a serotype that usually causes invasive infections, was specifically identified for any serogroup C1 \textit{Salmonella} isolate showing no citrate utilization. Thus \textit{S. Choleraesuis} was separated from the rest of the serogroup C isolates for statistical analysis and discussion throughout this study unless otherwise stated. Demographic data and final diagnoses of the patients with culture-confirmed \textit{Salmonella} infections were obtained through chart review and analysed.

The antimicrobial susceptibility of these isolates was investigated by a standard disc-diffusion method for non-blood isolates and by a broth-microdilution method for blood isolates.\textsuperscript{18,19} The antimicrobial agents examined included ampicillin, cefixime, ceftriaxone, chloramphenicol, ciprofloxacin and trimethoprim/sulfamethoxazole. Susceptibility and resistance were defined according to the criteria suggested by the NCCLS.\textsuperscript{18,19} The isolates in the ‘intermediate’ category were deemed as ‘resistant’ in this study.

Investigation of ceftriaxone resistance

To study the resistance mechanism, clinical isolates of \textit{Salmonella} with resistance to ceftriaxone were examined retrospectively. For each patient, only the first ceftriaxone-resistant isolate was included for study even if multiple isolates were available. MICs of cefotaxime, ceftazidime, cefepime (all with or without the presence of clavulanic acid) and cefoxitin were determined by a standard broth-microdilution method.\textsuperscript{19}

Ceftriaxone resistance genes were amplified by PCR using consensus primer sets previously described for detecting \textit{bla}\textsubscript{TEM}, \textit{bla}\textsubscript{SHV}, \textit{bla}\textsubscript{CTX,M} and \textit{ampC} genes.\textsuperscript{10,20–22} PCR products were purified by using the Microcon\textsuperscript{R} PCR Centrifugal Filter Devices (Millipore Corporation, Bedford, MA, USA) and sequenced by an ABI 377 automatic sequencer (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). The nucleotide sequences obtained were compiled and analysed using the Lasergene software (DNASTAR, Inc., Madison, WI, USA). The search for homologous sequences was conducted in the GenBank database using the Basic Local Alignment Search Tool (BLAST) through the Internet (http://www.ncbi.nlm.nih.gov/BLAST/).

To localize the \textit{ampC} gene, a pair of primers (AmpC-F, 5'-CAAGTTGATTCTCTGGACTCT; and AmpC-R, 5'-CTCATCGTCAGTTATGGACGT) was first used to amplify the full-length gene. The PCR product was labelled with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) and used as a probe in DNA–DNA hybridization.\textsuperscript{23} The plasmid DNA was prepared by a method described previously and used as the template in the hybridization.\textsuperscript{24} To decipher the genetic relatedness of the ceftriaxone-resistant isolates, infrequent-restriction-site PCR was used to genotype the isolates as described previously.\textsuperscript{25}

Statistical analysis

The \(\chi^2\) test and Student’s \(t\)-test were used to determine the significance of differences. A difference was considered statistically significant with a \(P\) value < 0.05.

Results

Prevalence

A total of 3635 \textit{Salmonella} isolates, including 3592 (98.8%) non-typhoid \textit{Salmonella}, were identified during the study period. The annual isolate number of \textit{Salmonella} fluctuated between 681 and 774 (average, 727). Compared with the annual number of

Figure 1. Distribution of annual isolate numbers of \textit{Salmonella} serogroups in CGMH, 1999–2003. Open squares, serogroup B; open diamonds, serogroup D; open circles, serogroup C; crosses, serogroup E; open triangles, \textit{Salmonella enterica} serotype Choleraesuis; filled squares, serotype Typhi; asterisks, serotype Paratyphi; filled circles, other \textit{Salmonella} spp.
total bacteria isolated from this laboratory (49,778–66,370; average, 58,799), the proportion of Salmonella isolates remained fairly stable, at between 1.1–1.5% (average, 1.24%) per year. Figure 1 shows the annual isolate number of each serogroup over the study period. Serogroup B accounted for 55.6% (range, 44.6–66.1%) of all Salmonella isolates and was the most prevalent; however, an apparent decrease from 512 to 304 (average, 404) isolates per year was observed (P < 0.0000005). Serogroup D ranked the second, and in contrast to serogroup B, the annual isolate number increased significantly from 105 to 155 (average, 137), corresponding to 13.6–22.8% (average, 18.8%) of the total Salmonella isolates (P < 0.0000005). The third most frequently isolated Salmonella belonged to serogroup C, with an annual isolate number varying between 92 (11.9%) and 132 (19.4%) (average, 110 or 15.1%). S. Choleraesuis caused the fourth largest population, with a trend of isolation similar to that observed in serogroup C. There were 23–67 (average, 43) S. Choleraesuis isolates each year during the study period, comprising 6.0% (range, 3.0–9.8%) of the total Salmonella isolates. As for S. Typhi, the annual isolate number decreased from 16 to 4, with an average of 7, which represented only 1.0% of the total Salmonella isolates.

**Age distribution and clinical infections**

When the age of patients was compared, the largest population was infants <1 year of age (32%); the prevalence increased gradually following birth (0.6%) and peaked at the 12th month of life (7.1%). The trend decreased abruptly after infancy, and children of the pre-school ages (between 1–6 years old) formed another large group (35%). The trend declined continuously and flattened during school ages throughout the adult years (1–5 cases per year; average, 3 cases), although a slight increase was noted during the 7th decade of life (3–8 cases per year; average, 6 cases). The trend was generally the same among various non-typhoid Salmonella serogroups, although infant patients <1 year of age were more likely to be infected by serogroup B Salmonella than children in other age groups (P < 0.0001).

Gastroenteritis (70%) was the most common infection caused by non-typhoid Salmonella, followed by bacteremia (20%), skin infections (5.4%), urinary tract infections (2.5%) and infections of other sites (2.1%). Like S. Typhi, most (93.1%) of the S. Choleraesuis caused extra-intestinal infections, including bacteremia (72%). Second to S. Choleraesuis, 27.5% of the serogroup D isolates were derived from blood. A significant increase in serogroup D bacteremic isolates was found over the study period (25/105 in 1999 versus 55/155 in 2003, P < 0.05).

**Antimicrobial resistance**

For non-typhoid Salmonella, the average resistance rates to conventional antibiotics (50% to ampicillin, 56% to chloramphenicol and 37% to trimethoprim/sulfamethoxazole) were much higher than those to other drugs, such as 2.2% to cefixime, 1.5% to ceftriaxone and 5.0% to ciprofloxacin, during the study period. Resistance to ceftriaxone increased significantly from 0.8% in 1999 to 1.5% in 2003 (0.8–2.1%; average, 1.5%) (P < 0.000001). When different groups of Salmonella were compared (Figure 2), the highest resistance was detected in S. Choleraesuis; during the study period, resistance increased significantly in terms of ampicillin (76–91%; average, 87%), chloramphenicol (60–91%; average, 85%), trimethoprim/sulfamethoxazole (50–88%; average, 77%) and ciprofloxacin (0–69%; average, 58%). Isolates of serogroup D remained the most susceptible, with the highest resistance rate of 15% to trimethoprim/sulfamethoxazole in 2003. For the other Salmonella, the pattern of antimicrobial resistance remained relatively constant over the study period (Figure 2). Results for serogroup D Salmonella during 1999–2002 in Figures 1 and 2 and the trend of ciprofloxacin resistance in S. Choleraesuis during 2000–2003 in Figure 2 have previously been included in other studies.26,27 The data are retained in the current report as a comparison with the most up-to-date information and to maintain the integrality of the whole study.

Regarding S. Typhi and S. Paratyphi, all isolates were susceptible to the above-mentioned antibiotics, except the one ampicillin-resistant S. Typhi and one trimethoprim/sulfamethoxazole-resistant S. Paratyphi C1 that were isolated in 1999 and 2001, respectively.

**Mechanism of ceftriaxone resistance**

Resistance to ceftriaxone in non-typhoid Salmonella has been emerging in Taiwan. To uncover the resistance mechanism, a total of 22 non-repetitive ceftriaxone-resistant isolates, including 12 serogroup B, six serogroup C, three serogroup D and one serogroup E isolates, were retrospectively examined (Table 1). The remaining ceftriaxone-resistant isolates from the other 14 patients were not retrievable for study. By PCR and DNA sequencing, blacMY-2 was found in 14 isolates, including 10 serogroup B and four serogroup C. Genes encoding extended-spectrum β-lactamases (ESBLs), including CTX-M-3 (n = 6), SHV-12 (n = 1) and SHV-2a (n = 1), were found in another eight isolates. Six isolates also contained blaTEM-1, which coexisted with blactMX-3 (n = 2) or blacMY-2 (n = 2). The phenotypic presentation of these genes was also reflected by the MIC results (Table 1). Among the six isolates that showed intermediate resistance to ceftriaxone (MIC, 16–32 mg/L), five carried blacMY-2. Southern-blot hybridization showed that the ampC gene was located on plasmids of 6.6–160 kb in size (Table 1). Diverse serotypes and genotypes were found among these ceftriaxone-resistant isolates (Table 1).

Resistance to chloramphenicol and trimethoprim/sulfamethoxazole was found in 12 isolates, respectively. One isolate of S. Choleraesuis was resistant to all six antibiotics tested, with the MICs of ceftriaxone and ciprofloxacin both at 16 mg/L. The resistance mechanism of the isolate has been reported in detail recently.28 The remaining 21 isolates were susceptible to ciprofloxacin.

**Discussion**

During the study period, the annual isolate number of Salmonella remained constant at 1.1–1.5% of total bacterial isolates, but the trend varied among different serogroups. Serogroup B remained the most frequent Salmonella serogroup in the locality, but one encouraging observation is that the annual prevalence of this serogroup decreased by half compared with that in 1995.3 In contrast, a nearly four-fold increase was observed in the prevalence of serogroup D isolates during the same period. Although serotyping data were not available for these isolates, according to the data from the Center for Disease Control in Taiwan and our
previous studies, S. Typhimurium and S. Enteritidis are the most predominant serotypes among serogroups B and D Salmonella isolates, respectively. If this trend continues, the prevalence of non-typhoid serogroup D-induced salmonellosis in Taiwan will soon surpass that of serogroup B infection, as has occurred in the USA and Europe. An even more worrying situation is that a substantial proportion of serogroup D isolates were derived from blood over the years. It appears that the increase in serogroup D Salmonella infections, bacteraemia in particular, has not reached a plateau. Such an escalating trend must be monitored very carefully.

The finding that approximately one-third of salmonellosis occurred in infants <1 year old is striking. Compared with the proportion (15%) in this age group reported in the USA, our data indicate a far greater salmonellosis problem in young children in Taiwan. The spectrum of infections caused by Salmonella in Taiwan is very similar to those previously recognized, i.e. gastroenteritis is the most common clinical presentation. However, the overall rate of bacteraemia (20%) is much higher than those reported in the USA. This may be due in part to the fact that patients with severe infections are more likely to be referred to our hospital as a tertiary-care hospital. Another possible explanation may be that infections caused by S. Choleraesuis have been relatively more rampant in Taiwan than in other countries.

S. Choleraesuis is known as the most invasive serotype among non-typhoid Salmonella and usually causes bacteraemia — 72% in our study and 63% in the USA — or extra-intestinal focal infections without overt diarrhoeal illness in humans.

The most important finding of this study is the emergence of ceftriaxone resistance among Salmonella isolates, an event also noticed in other countries. Molecular analysis indicated that the majority of ceftriaxone resistance was due to the production of CMY-2 (64%) and CTX-M-3 (27%) β-lactamases. The only

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Figure 2. Secular trends in antimicrobial resistance to different antimicrobial agents among various Salmonella serogroup isolates in CGMH, 1999–2003. Open squares, ampicillin; filled squares, chloramphenicol; open circles, trimethoprim/sulfamethoxazole; open diamonds, ciprofloxacin; crosses, cefixime; filled circles, ceftriaxone.
Table 1. Characterization of the 22 Salmonella isolates that were resistant to ceftriaxone

<table>
<thead>
<tr>
<th>Salmonella</th>
<th>Year of isolation</th>
<th>Specimen</th>
<th>Genotype(^a)</th>
<th>β-Lactamase</th>
<th>Plasmid containing bla(_{\text{CMY-2}})</th>
<th>Antimicrobial resistance to CHL/SXT/CFM/CIP(^b)</th>
<th>MIC(^c) (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serogroup B</strong></td>
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<tr>
<td>Gloucester</td>
<td>2000</td>
<td>stool</td>
<td>B-1</td>
<td>CMY-2</td>
<td>80</td>
<td>R/R/S/R/S</td>
<td>128 128 64 64 64 64 8 128</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>2001</td>
<td>stool</td>
<td>B-2</td>
<td>CMY-2</td>
<td>120</td>
<td>R/R/R/S</td>
<td>32 128 64 64 64 16 4 128</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>2001</td>
<td>stool</td>
<td>B-3</td>
<td>CMY-2</td>
<td>90</td>
<td>R/S/R/S</td>
<td>64 128 64 64 64 1 1 128</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>2001</td>
<td>stool</td>
<td>B-4</td>
<td>CMY-2</td>
<td>80</td>
<td>S/S/R/S</td>
<td>16 16 16 64 64 1 1 128</td>
</tr>
<tr>
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<td>2002</td>
<td>stool</td>
<td>B-5</td>
<td>CMY-2</td>
<td>90</td>
<td>R/R/R/S</td>
<td>64 128 64 64 64 1 1 32</td>
</tr>
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<td>stool</td>
<td>B-6</td>
<td>CMY-2/TEM-1</td>
<td>80</td>
<td>R/R/R/S</td>
<td>64 16 16 64 64 1 1 128</td>
</tr>
<tr>
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<td>stool</td>
<td>B-7</td>
<td>CMY-2</td>
<td>90</td>
<td>S/S/R/R</td>
<td>16 128 64 64 64 1 1 128</td>
</tr>
<tr>
<td>Mons</td>
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<td>stool</td>
<td>B-8</td>
<td>CMY-2</td>
<td>110</td>
<td>R/R/R/S</td>
<td>&gt;128 &gt;128 &gt;128 &gt;128 &gt;128 16 8 128</td>
</tr>
<tr>
<td>Mons</td>
<td>2002</td>
<td>blood</td>
<td>B-9</td>
<td>CTX-M-3/TEM-1</td>
<td>ND(^d)</td>
<td>R/R/R/S</td>
<td>&gt;128 128 1 32 8 &gt;128 8 32</td>
</tr>
<tr>
<td>Kimuienza</td>
<td>2002</td>
<td>stool</td>
<td>B-10</td>
<td>CMY-2</td>
<td>80</td>
<td>S/R/R/S</td>
<td>32 32 32 64 64 1 1 128</td>
</tr>
<tr>
<td>Schleissheim</td>
<td>2002</td>
<td>blood</td>
<td>B-12</td>
<td>CTX-M-3/TEM-1</td>
<td>ND(^d)</td>
<td>S/R/R/S</td>
<td>&gt;128 128 1 8 1 32 1 4</td>
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<td><strong>Serogroup C1</strong></td>
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<tr>
<td>Redba</td>
<td>2000</td>
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<td>C1-1</td>
<td>CMY-2</td>
<td>160</td>
<td>R/S/R/S</td>
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<tr>
<td>Redba</td>
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<td>stool</td>
<td>C1-2</td>
<td>CMY-2</td>
<td>90</td>
<td>S/S/R/S</td>
<td>64 64 64 128 128 1 1 &gt;128</td>
</tr>
<tr>
<td>Choleraesuis(^e)</td>
<td>2002</td>
<td>blood</td>
<td>C1-3</td>
<td>CMY-2/TEM-1</td>
<td>140</td>
<td>R/R/R/R</td>
<td>16 8 8 32 32 1 1 64</td>
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<tr>
<td>Choleraesuis(^e)</td>
<td>2002</td>
<td>stool</td>
<td>C1-4</td>
<td>CTX-M-3/TEM-1</td>
<td>ND(^d)</td>
<td>S/S/R/S</td>
<td>&gt;128 128 1 16 1 128 4 32</td>
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<tr>
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<td>2001</td>
<td>stool</td>
<td>C2-1</td>
<td>CMY-2</td>
<td>90</td>
<td>S/S/R/S</td>
<td>64 128 64 64 64 1 1 128</td>
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<tr>
<td>Albany</td>
<td>2002</td>
<td>stool</td>
<td>C2-2</td>
<td>CTX-M-3</td>
<td>ND(^d)</td>
<td>R/R/S/R</td>
<td>&gt;128 128 1 8 1 16 1 32</td>
</tr>
<tr>
<td><strong>Serogroup D</strong></td>
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<tr>
<td>Enteritidis</td>
<td>2001</td>
<td>stool</td>
<td>D-1</td>
<td>SHV-2a</td>
<td>ND(^d)</td>
<td>S/R/S/R</td>
<td>64 128 1 64 1 16 1 4</td>
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<td>Enteritidis</td>
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<td>stool</td>
<td>D-2</td>
<td>SHV-12</td>
<td>ND(^d)</td>
<td>R/R/S/R</td>
<td>16 32 1 &gt;128 1 16 1 4</td>
</tr>
<tr>
<td>Enteritidis</td>
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<td>blood</td>
<td>D-3</td>
<td>CTX-M-3</td>
<td>ND(^d)</td>
<td>S/R/S/R</td>
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<td><strong>Serogroup E</strong></td>
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<td>wound</td>
<td>E-1</td>
<td>CTX-M-3/TEM-1</td>
<td>ND(^d)</td>
<td>R/R/R/S</td>
<td>&gt;128 128 1 16 1 64 1 32</td>
</tr>
</tbody>
</table>

\(^a\)Genotypes were determined by infrequent-restriction-site PCR. Results are expressed by the respective serogroup, followed by the Arabic numerals showing the actual genotypes.

\(^b\)CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; CFM, cefixime; CIP, ciprofloxacin. Antimicrobial resistance was examined by the standard disc diffusion method.\(^18\)

\(^c\)CRO, ceftriaxone; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; CLA, clavulanic acid.

\(^d\)ND, not done.

\(^e\)The clinical presentation and resistance mechanisms have been reported in detail previously, due to the unique nature of the two isolates.\(^15,28\)
two SHV-type ESBLs were found in S. Enteritidis isolates. Diverse genotypes suggest that horizontal transfer of resistance genes plays a role in the increasing ceftriaxone resistance. We have further verified this hypothesis by confirming that all the ampC genes were located on plasmids. Earlier studies showed that, in Salmonella, other types of ESBLs, such as CTX-M, SHV and TEM, are also plasmid-mediated. The majority of these ceftriaxone-resistant isolates are usually co-resistant to many other antibiotics. This is in accordance with an earlier concept that multidrug resistance of non-typhoid Salmonella involves the acquisition and accumulation of plasmid-mediated resistance determinants and/or spreading of mobile elements, such as transposons. The ability of these mobile elements to transfer within or between bacterial species is associated with the rapid spread of antimicrobial resistance among the Enterobacteriaceae, including Salmonella.

Despite an increasing number of reports describing the production of extended-spectrum cephalosporinas among Salmonella, many clinical microbiological laboratories have not included Salmonella in their routine screening for such resistance. We found in the present study that among the six isolates showing intermediate resistance to ceftriaxone (MIC, 16–32 mg/L), live carried blaoctam-2 and one had blashv-12, indicating that extended-spectrum cephalosporinas may not be the appropriate drugs to treat patients infected with these organisms. Failure to detect and report Salmonella isolates capable of producing β-lactamases may lead to inappropriate choice of antibiotics in the clinical setting when treating invasive salmonellosis. The inclusion of screening tests for ESBLs and AmpC β-lactamases in routine susceptibility testing for Salmonella appears feasible.

Previous studies have addressed the health impact of drug resistance in types of zoonotic salmonellae, and suggested that the drug resistance may be associated with increased illness and death rates. The growing incidence of antimicrobial-resistant Salmonella infections therefore represents a serious threat to public health. Since the resistance displayed by Salmonella generally reflects the environment in which the organism thrives, to curb such resistance problems, immediate action, including restriction of the usage of extended-spectrum cephalosporinas and continuous surveillance for resistant Salmonella, is necessary.

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