Increased Resistance to Multiple Antimicrobials and Altered Resistance Gene Expression in CMY-2-Positive Salmonella enterica following a Simulated Patient Treatment with Ceftriaxone

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Salmonellosis is one of the most common causes of food-borne disease in the United States. Increasing antimicrobial resistance and corresponding increases in virulence present serious challenges. Currently, empirical therapy for invasive Salmonella enterica infection includes either ceftriaxone or ciprofloxacin (E. L. Hohmann, Clin. Infect. Dis. 32:263–269, 2001). The bla<sub>CMY-2</sub> gene confers resistance to ceftriaxone, the antimicrobial of choice for pediatric patients with invasive Salmonella enterica infections, making these infections especially dangerous (J. M. Whichard et al., Emerg. Infect. Dis. 11:1464–1466, 2005). We hypothesized that bla<sub>CMY-2</sub>-positive Salmonella enterica would exhibit increased MICs to multiple antimicrobial agents and increased resistance gene expression following exposure to ceftriaxone using a protocol that simulated a patient treatment in vitro. Seven Salmonella enterica strains survived a simulated patient treatment in vitro and, following treatment, exhibited a significantly increased ceftriaxone MIC. Not only would these isolates be less responsive to further ceftriaxone treatment, but because the bla<sub>CMY-2</sub> genes are commonly located on large, multidrug-resistant plasmids, increased expression of the bla<sub>CMY-2</sub> gene may be associated with increased expression of other drug resistance genes located on the plasmid (N. D. Hanson and C. C. Sanders, Curr. Pharm. Des. 5:881–894, 1999). The results of this study demonstrate that a simulated patient treatment with ceftriaxone can alter the expression of antimicrobial resistance genes, including bla<sub>CMY-2</sub> and floR in S. enterica serovar Typhimurium and S. enterica serovar Newport. Additionally, we have shown increased MICs following a simulated patient treatment with ceftriaxone for tetracycline, amikacin, ceftriaxone, and cefepime, all of which have resistance genes commonly located on CMY-2 plasmids. The increases in resistance observed are significant and may have a negative impact on both public health and antimicrobial resistance of Salmonella enterica.

Invasive bloodstream infection and hospitalization as a result of Salmonella enterica infection is associated with antimicrobial resistance. In patients with Salmonella enterica serovar Typhimurium infection, the association between resistance, invasive infection, and hospitalization is particularly strong (7, 9, 20). Therefore, not only does multiple-drug-resistant Salmonella enterica contribute to increased morbidity and mortality, it also contributes to increased health care costs. Isolates utilized in this study include Salmonella Typhimurium and Salmonella enterica serovar Newport strains, all of which have a plasmid containing the bla<sub>CMY-2</sub> and floR genes. While bla<sub>CMY-2</sub>-encoded β-lactamase that confers resistance to β-lactam antimicrobials, the floR gene encodes a phenicol-specific efflux pump which confers resistance to phenicol antimicrobials, such as chloramphenicol (2). The bla<sub>CMY-2</sub>-encoded AmpC β-lactamase enzymes found in these strains are divergent from traditional extended-spectrum β-lactamases in that bla<sub>CMY-2</sub>-encoded AmpC β-lactamase enzymes are not inhibited by clavulanic acid.

A series of Salmonella Typhimurium and Salmonella Newport strains were subjected to a simulated patient treatment regimen with ceftriaxone in vitro. We hypothesized that bla<sub>CMY-2</sub>-positive
**Salmonella enterica** would exhibit increased MICs to multiple antimicrobial agents and increased resistance gene expression following exposure to ceftriaxone using a protocol that simulated patient treatment. Here, we show a statistically significant increase in MICs to ceftriaxone, cefepime, amikacin, and tetracycline following treatment. Additionally, the expression of both **bla** and **floR** was influenced by exposure to ceftriaxone.

**MATERIALS AND METHODS**

**Bacterial isolates and growth conditions.** Four *Salmonella enterica* serovar Typhimurium strains and three *Salmonella enterica* serovar Newport strains were utilized in this study. Strains utilized were obtained from a laboratory stock of CMY-2-positive *Salmonella* strains which were screened for the ability to survive a simulated patient treatment with ceftriaxone. The strains had been previously obtained from human stool, porcine liver, bovine liver, and bovine intestine. Those designated pretreatment isolates were the strains in their original state. Isolates labeled posttreatment were pretreatment isolates which had been exposed to ceftriaxone using a protocol that simulated patient treatment with ceftriaxone. Following the simulated patient treatment, these isolates were designated posttreatment isolates. Isolates designated posttreatment with ceftriaxone pressure were posttreatment isolates grown with 8 µg/ml ceftriaxone prior to MIC and gene expression assays. Isolates retained their designated strain numbers with the addition of the treatment designation throughout the experiment. All isolates were stored at −80°C until further use.

**Replication of patient treatment in vitro.** The pharmacokinetics of a 2-g ceftriaxone adult dose was simulated in vitro using the following protocol. Bacterial cells were grown on Mueller-Hinton agar (MH; Becton, Dickinson, and Company, Sparks, MD) at 37°C for 18 h. A colony from each plate was used to inoculate 10 ml MH broth (BD) in a 14-ml Falcon tube (BD) which was grown at 37°C for 2 h on an orbital shaker (New Brunswick Scientific, Edison, NJ) at 100 rpm. The simulated patient treatments with ceftriaxone (Sigma-Aldrich, St. Louis, MO) were all performed with 50 ml culture in 150-ml flasks while shaking at 100 rpm and 37°C. Briefly, the treatment concentrations, all with ceftriaxone (Sigma-Aldrich), were as follows: 257 µg/ml for 45 min, 192 µg/ml for 1 h, 154 µg/ml for 2 h, and 60 µg/ml for 18 h. The concentrations used were based off the mean plasma concentration of ceftriaxone for an adult receiving a 2-g intravenous dose (14).

**Antimicrobial susceptibility testing.** MICs were determined by broth microdilution as described by the Clinical and Laboratory Standards Institute (CLSI) standard protocol (4, 5). The following antimicrobials were used: ampicillin (AMP; Fisher Scientific, Fair Lawn, NJ), cephalothin (CEF; Sigma-Aldrich, St. Louis, MO), ceftriaxone (CRO; Sigma-Aldrich), cefepime (FEP; USP, Rockville, MD), gentamicin (GEN; Fisher Scientific), amikacin (AMK; Fisher Scientific), tetracycline (TET; MP Biomedicals, Solon, OH), chloramphenicol (CHL; MP Biomedicals), ciprofloxacin (CIP; LKT Laboratories, St. Paul, MN), and piperacillin-tazobactam (TZP; MP Biomedicals/Chem-Impex International, Wood Dale, IL). *Escherichia coli* ATCC 25922 was used as a quality control organism in all antimicrobial susceptibility assays. All assays were run at least twice in duplicate.

**RNA isolation and quantitative reverse transcriptase PCR (qRT-PCR).** Total RNA was isolated from liquid culture with an optical density at 600 nm (OD600) of 0.4 for all *Salmonella* Typhimurium and *Salmonella* Newport isolates using the Promega SV total RNA isolation system (Promega Corporation, Madison, WI) according to the manufacturer’s instructions. An additional DNase treatment was performed using RNase-free DNase (Promega Corporation). The concentration and quality of the RNA in each sample were determined by measuring the absorbance at 230, 260, and 280 nm. Reverse transcription reactions were performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s recommendations.

Relative expression levels of **bla** and **floR** were determined by performing qRT-PCR on DNA obtained following the reverse transcription reactions. Amplification was performed with consensus primers (Table 1) for **bla** and **floR** genes as previously described (19). The qPCR amplifications were performed by combining 10 µl of 10-ng/µl cDNA with a 15 µl mixture of SYBR green Supermix (Bio-Rad, Hercules, CA) and 10 nM primers. The fluorescence signal was detected by a MiniOpticon real-time PCR instrument (Bio-Rad). The **rea** gene was used as an internal control for all copy number determinations (19). Data shown are the means from three replicates. DNA fold change of the **bla** and **floR** genes was determined using the Pfaffl method (15).

**Statistical analysis.** Data are presented as means and standard deviations. Statistical comparisons of MIC data were made using the Kruskal-Wallis test with Dunn’s post hoc test where appropriate (8). Gene expression data were statistically analyzed using the independent Student t test (8). A P value of less than 0.05 was considered significant.

**RESULTS**

To determine if a simulated patient treatment had an effect on **bla** and **floR** gene expression and resistance to ceftriaxone, qRT-PCR and MIC assays were performed. The data in Fig. 1 show the relative change in **bla** and **floR** gene expression following exposure to ceftriaxone using a protocol that simulated patient treatment in vitro. Presence of the extended-spectrum β-lactamase gene **bla** was detected in all isolates. *Salmonella* Typhimurium strains 043 and 032 and *Salmonella* Newport strain 023 demonstrated a significant increase (2.1- to 10.2-fold) in **bla** gene expression following a simulated patient treatment with ceftriaxone (Fig. 1A). However, *Salmonella* Newport 044 and 016 and *Salmonella* Typhimurium 030 showed a significant decrease (0.5- to 0.6-fold) in **bla** expression, while *Salmonella* Typhimurium 047 exhibited no change (1.0-fold) in posttreatment isolates (Fig. 1A). MICs increased significantly in posttreatment isolates (155 µg/ml) relative to those of pretreatment isolates (52 µg/ml) (Table 2).

Examination of the effect of continued ceftriaxone pressure on

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**TABLE 1 Primers used for qRT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Nucleotide sequence (5’ to 3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>bla</strong>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>F</td>
<td>GCC GTG TTG GCC GGC GAT G</td>
<td>369</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAG GGG AAC GCT AAT CCA G</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>floR</strong></td>
<td>F</td>
<td>CGA GCA GCT TGG TCT TCA AC</td>
<td>142</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CGT AGA TGA CGA CAC CCT CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rea</strong></td>
<td>F</td>
<td>TAC GCA ACA TCA CGG CAA TC</td>
<td>108</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GTA TGA TGC GCC AGG CGA TG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
gene expression and resistance to ceftriaxone and cefepime was assessed by qRT-PCR and MICs. Salmonella Newport 044 and 023 and Salmonella Typhimurium 043, 047, 030, and 032 posttreatment isolates all displayed statistically significant increases (1.4- to 22.2-fold) in \( \text{bla}_{\text{CMY-2}} \) expression when grown in 8 \( \mu \text{g/ml} \) ceftriaxone (Fig. 1B). Expression of \( \text{bla}_{\text{CMY-2}} \) increased in Salmonella Newport 016 when grown in ceftriaxone; however, the increase was not significant (1.2-fold) (Fig. 1B). The increase in \( \text{bla}_{\text{CMY-2}} \) expression correlated well with a significant increase in MIC in posttreatment isolates with ceftriaxone pressure (162 \( \mu \text{g/ml} \)) relative to that in pretreatment isolates (52 \( \mu \text{g/ml} \) (Table 2). The MICs to another extended-spectrum cephalosporin, cefepime, also showed a statistically significant increase in posttreatment isolates (128 \( \mu \text{g/ml} \)) relative to that in pretreatment isolates (91 \( \mu \text{g/ml} \)). Interestingly, MICs to cefepime decreased in posttreatment isolates with ceftriaxone pressure relative to that in pretreatment isolates; however, the decrease was not statistically significant (Table 2). Further investigation into the decrease in cefepime MIC is necessary in order to determine the mechanism behind the decreased MIC observed.

The effect of a simulated patient treatment with ceftriaxone on chloramphenicol resistance and \( \text{floR} \) expression was determined via MIC and qRT-PCR assays. The expression of \( \text{floR} \) in the posttreatment isolates with ceftriaxone pressure increased significantly (1.6- to 21.0-fold) in Salmonella Newport 044, 016, and 023 and Salmonella Typhimurium 047 (Fig. 1C). Decreased expression (0.1- to 0.2-fold) was seen in Salmonella Typhimurium 030 and 032, while no significant change (0.8-fold) was observed in Salmonella Typhimurium 043 (Fig. 1C). Chloramphenicol MICs increased in the posttreatment isolates with ceftriaxone pressure (186 \( \mu \text{g/ml} \)) relative to that in the pretreatment isolates (150 \( \mu \text{g/ml} \)); however, the increase was not statistically significant.

Following a simulated patient treatment with ceftriaxone, significant increases in MICs were observed for ceftriaxone, cefepime, amikacin, and tetracycline (Table 2). It is important to note that the increase in MICs observed was not due to differences in growth rate between the pretreatment and posttreatment isolates (data not shown). There was no statistically significant increase in MIC for ampicillin, cephaplatin, gentamicin, ciprofloxacin, or piperacillin-tazobactam (Table 2). Tetracycline MICs exhibited a pattern similar to that for ceftriaxone, with statistically significant increases in posttreatment isolates (107 \( \mu \text{g/ml} \)) and posttreatment isolates with ceftriaxone pressure (112 \( \mu \text{g/ml} \)) relative to the MICs in pretreatment isolates (79 \( \mu \text{g/ml} \) (Table 2).

![FIG 1 Posttreatment \( \text{bla}_{\text{CMY-2}} \) (A), posttreatment-with-ceftriaxone-pressure \( \text{bla}_{\text{CMY-2}} \) (B), and \( \text{floR} \) (C) gene expression (fold change) among S. enterica serovar Newport 044, 016, and 023 and S. enterica serovar Typhimurium 043, 047, 030, and 032 by qRT-PCR, relative to the expression of their respective pretreatment isolates. Expression of \( \text{bla}_{\text{CMY-2}} \) and \( \text{floR} \) was normalized to \( \text{recA} \) for each sample. Gene expression data were statistically analyzed using independent Student t tests. Bars show the means of three replicates. Dashed lines show a fold change of 1, error bars indicate the standard deviation, and asterisks show significant differences (\( P < 0.05 \)).

**TABLE 2** Average MICs for *Salmonella* Typhimurium and *Salmonella* Newport isolates used in this study

<table>
<thead>
<tr>
<th>Breakpoint or isolate group</th>
<th>MIC (( \mu \text{g/ml} ))a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMP</td>
</tr>
<tr>
<td><strong>Breakpoint</strong></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>32</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>1,836 (418)</td>
</tr>
<tr>
<td>Posttreatment with CRO pressure</td>
<td>( \geq 2,000 ) (0)</td>
</tr>
<tr>
<td><strong>Isolate group</strong></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>ND</td>
</tr>
<tr>
<td>Posttreatment</td>
<td></td>
</tr>
</tbody>
</table>

a Values represent means (standard deviations) from 7 strains utilized in this study. AMP, ampicillin; CEF, cephaplatin; CRO, ceftriaxone; FEP, cefepime; AMK, amikacin; GEN, gentamicin; TET, tetracycline; CHL, chloramphenicol; CIP, ciprofloxacin; TZP, piperacillin-tazobactam. Statistical comparisons of MIC data were made using the Kruskal-Wallis test with Dunn’s post hoc test where appropriate. *, \( P < 0.05 \); ND, not determined.
Increased Resistance following Ceftriaxone Treatment

We have shown that a simulated patient treatment with ceftriaxone altered \( \text{bla}_{\text{CMY-2}} \) and \( \text{floR} \) expression as well as the phenotypic resistance of multiple isolates. Relative \( \text{bla}_{\text{CMY-2}} \) expression increased significantly in six of seven posttreatment isolates with ceftriaxone pressure, with one isolate showing an increase that was not statistically significant (Fig. 1B). There was no difference in growth between the pretreatment and posttreatment isolates, as determined by growth curve assays, which indicated that the phenotypic change in resistance was not due to an alteration in growth parameters. Interestingly, of the posttreatment isolates grown without ceftriaxone pressure, just three of the seven isolates exhibited a significant increase in \( \text{bla}_{\text{CMY-2}} \) gene expression (Fig. 1A). Compared to \( \text{bla}_{\text{CMY-2}} \) expression in the presence of ceftriaxone, these data suggest that increases in \( \text{bla}_{\text{CMY-2}} \) expression are short-lived and require the presence of ceftriaxone.

The increase in \( \text{bla}_{\text{CMY-2}} \) expression observed in the posttreatment isolates with ceftriaxone pressure correlated with the MIC data. The MICs to cefepime also increased following treatment with ceftriaxone (Table 2), a result which indicated that a common mechanism likely conferred resistance to both of these cephalosporins. Extended-spectrum cephalosporin resistance, mediated by \( \text{bla}_{\text{CMY-2}} \) genes, is the major mechanism of cephalosporin resistance among \emph{Salmonella enterica} strains (1, 6). Resistance to \( \beta \)-lactam antimicrobials, such as ceftriaxone, is linearly correlated with the lactamase level over a large range, and resistance to \( \beta \)-lactams can be achieved by increasing enzyme levels (22). Thus, with increased \( \text{bla}_{\text{CMY-2}} \) expression, increased resistance would be expected. Our data indicate that therapeutic concentrations of ceftriaxone can induce increased \( \text{bla}_{\text{CMY-2}} \) expression. This finding builds upon previous studies with \emph{Enterobacteriaceae} which have shown inducible AmpC expression in response to \( \beta \)-lactam antimicrobials (10, 12).

In a recent study, \emph{Salmonella Typhimurium} lineages carrying a \( \text{bla}_{\text{TEM-1}} \) \( \beta \)-lactamase gene, which is similar to the \( \text{bla}_{\text{CMY-2}} \) gene in function, were allowed to develop resistance to progressively increased concentrations of cephalothin and cefaclor (19). As the lineages developed resistance to higher antimicrobial concentrations, amplification of the \( \text{bla}_{\text{TEM-1}} \) gene was found to be the primary and most common resistance mechanism (19). Though this study does not present evidence of gene amplification as a resistance mechanism, increases in \( \text{bla}_{\text{CMY-2}} \) expression as a result of exposure to cephalosporins highlight an additional mechanism leading to increased resistance as a result of cephalosporin exposure. The data in the present study complement this work while employing extended-spectrum cephalosporins. The study on \( \text{bla}_{\text{TEM-1}} \)-mediated resistance utilized slowly increasing concentrations of antimicrobials not commonly used to treat invasive \emph{Salmonella enterica} infections; however, this study enlisted one of the drugs of choice for treatment of invasive infections. Additionally, the isolates were exposed to ceftriaxone in a manner which simulated patient infection and treatment. The simulated patient treatment with ceftriaxone closely mimics the mean plasma concentrations seen in both adult and pediatric patients (14, 18). Thus, this study contributes to the growing body of knowledge of \emph{Salmonella enterica} drug resistance and response to antimicrobial treatment.

Expression of \( \text{floR} \) was determined via qRT-PCR for pretreatment isolates and posttreatment isolates with ceftriaxone pressure. Statistically significant increases in expression of \( \text{floR} \) were observed in four of seven posttreatment isolates under ceftriaxone pressure, significant decreases in expression were observed in two posttreatment isolates under ceftriaxone pressure, and one isolate exhibited no significant change in \( \text{floR} \) expression (Fig. 1C). The MICs to chloramphenicol did increase in posttreatment isolates grown under ceftriaxone pressure relative to those in pretreatment isolates; however, the increase was not statistically significant (Table 2). Additional study is necessary to better understand the effect of ceftriaxone on \( \text{floR} \) gene expression and phenotypic resistance to chloramphenicol. Further, unlike the \( \text{bla}_{\text{CMY-2}} \) B gene product, an extended-spectrum \( \beta \)-lactamase enzyme in which resistance is directly proportional to the amount of enzyme present, the \( \text{floR} \) gene product is a multidrug efflux pump (2). Thus, \( \text{floR} \) regulation is more sensitive and small changes in expression can result in increased phenotypic resistance. Unlike with \( \text{bla}_{\text{CMY-2}} \) there is not a linear relationship between \( \text{floR} \) expression and phenotypic resistance (2).

It is important to note that the system utilized in this study does have some inherent limitations. Performing the simulated patient treatment \emph{in vitro} did allow duplication of the pharmacokinetics of ceftriaxone but did not incorporate any type of host immune response. The host immune response is an important factor in any infectious process, especially for intracellular pathogens such as \emph{Salmonella enterica}. Employing 7 strains, the data presented here characterize a response in a small sample of \emph{Salmonella enterica} strains. A larger sample would need to be studied before any generalizations could be made. This study focused on the interaction of \emph{Salmonella enterica} with ceftriaxone and its effect on phenotypic characteristics as well as expression of the \( \text{bla}_{\text{CMY-2}} \) and \( \text{floR} \) genes; however, moving this study into a model with a host immune response is necessary to better characterize the response \emph{in vivo}.

Though this initial study was performed \emph{in vitro} with a small number of \emph{Salmonella enterica} isolates, the data describe an important phenomenon. We intend to expand the study to include a larger number of \emph{Salmonella enterica} isolates which also contain the \( \text{bla}_{\text{CMY-2}} \) plasmid. Additionally, shifting the simulated patient infection and treatment to an animal model will provide necessary data for evaluating the effects of ceftriaxone treatment when combined with a functioning immune system. Since \emph{Salmonella enterica} is capable of host cell invasion and intracellular proliferation, an animal model is absolutely necessary to fully evaluate our initial findings. Performing gene expression arrays and, subsequently, QRT-PCR on additional resistance genes would give a more complete picture of the impact of a simulated patient treatment with ceftriaxone on \emph{Salmonella enterica}. Finally, sequencing of the \( \text{bla}_{\text{CMY-2}} \) plasmids and construction of plasmid maps would shed additional light on potential regulatory mechanisms involved in this phenomenon.

The fact that seven \emph{Salmonella enterica} isolates were able to survive a simulated patient treatment and then exhibit an increased ceftriaxone MIC is an important observation. Not only would these isolates be less responsive to further ceftriaxone treatment, but because the \( \text{bla}_{\text{CMY-2}} \) genes are commonly located on large, multidrug-resistant plasmids, increased expression of the \( \text{bla}_{\text{CMY-2}} \) B gene may also be associated with increased expression of other drug resistance genes located on the plasmid (10). The results of this study demonstrate that a simulated patient treatment with ceftriaxone can alter the expression of antimicrobial resis-
tance genes, including blaCMY-2 and floR in Salmonella Typhimurium and Salmonella Newport. Here, we show increased MICs following a simulated patient treatment with ceftriaxone for tetracycline, amikacin, ceftriaxone, and cefepime, all of which have resistance genes commonly located on CMY-2 plasmids. The increases in resistance observed are significant and may have a negative impact on both public health and antimicrobial resistance of Salmonella enterica.

ACKNOWLEDGMENTS

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