Ceftiofur-Resistant *Salmonella* Strains Isolated from Dairy Farms Represent Multiple Widely Distributed Subtypes That Evolved by Independent Horizontal Gene Transfer

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*Salmonella* is the leading cause of known food-borne bacterial infections in the United States, with an incidence rate of approximately 15 cases per 100,000 people. The rise of antimicrobial-resistant *Salmonella* strains has led to a decrease in the efficacy of these treatments (2). Currently, fluoroquinolones and broad-spectrum cephalosporins are the preferred drugs for treatment of adults and children, respectively, due to the low number of *Salmonella* isolates showing resistance to these drugs (2, 8, 9). However, the viability of these drugs may be diminishing as *Salmonella* strains producing β-lactamases conferring resistance to broad-spectrum cephalosporins have been isolated from clinical patients (11, 19, 38).

Of particular concern is the appearance of *Salmonella* strains with decreased susceptibility to ceftiofur (1, 5, 15, 38, 41). Ceftiofur is a broad-spectrum cephalosporin with wide-range activity against both gram-positive and gram-negative bacteria. It is the only broad-spectrum cephalosporin approved in the United States for treatment of dairy cattle (18). Ceftiofur is closely related to ceftriaxone, the drug of choice for treatment of children with invasive *Salmonella* infections (8, 9). Children under the age of 5 years account for 25% of all *Salmonella* infections in the United States (6). Beef and dairy products accounted for 10% of reported food-borne *Salmonella* outbreaks where a vehicle was identified (24). While a previous report suggested that infected cattle were the source of a ceftriaxone-resistant *Salmonella* infection in a child (12, 32), further data on the transmission and evolution of ceftiofur- and ceftriaxone-resistant *Salmonella* strains are needed.

The most common mechanism of cephalosporin resistance is the production of β-lactamases. Cephalosporins are semisynthetic antibiotics originally derived from cephalosporin C, a naturally occurring antimicrobial produced by *Cephalosporium acremonium*. Like other β-lactams, such as penicillin and ampicillin, cephalosporins act by targeting various penicillin-binding proteins that are essential for the synthesis of peptidoglycan, the major component of the bacterial cell wall (25). The antimicrobial activity of these antibiotics is due to the presence of a β-lactam ring. β-Lactamases confer resistance by hydrolyzing the β-lactam ring, producing β-amino acids with no antimicrobial activity (20). Broad-spectrum cephalosporins,

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like ceftiofur and ceftriaxone, are prescribed to treat Salmonella infections due to their increased activity against gram-negative bacteria and the presence of oxyimino side chains that provide increased ring stability in the presence of β-lactamases (18, 20).

Despite the effectiveness of broad-spectrum cephalosporins in combating Salmonella infections, resistant subtypes have emerged. Unlike other enterobacteria, Salmonella possesses no chromosomal β-lactamase gene (22). Instead, resistance to ceftiofur and ceftriaxone in Salmonella has been traced to a plasmid-encoded AmpC-like β-lactamase, CMY-2 (7, 38, 39). AmpC β-lactamases belong to class C of Ambler’s structural characterization, meaning that they are active-site serine β-lactamases and are typically encoded by chromosomal bla genes (20). Plasmid-borne ampC genes appear to be derived from chromosomal genes; for example, bla<sub>CMY-2</sub> is closely related to the chromosomal ampC gene found in Citrobacter freundii and has been found in plasmids carried by several Salmonella subtypes and other gastrointestinal bacteria (26, 39). Restriction fragment length polymorphism analysis and Southern blotting have shown that bla<sub>CMY-2</sub> resides on at least four different plasmids, termed types A, B, C, and D (5, 14, 39).

The goal of this study was to characterize a set of ceftiofur-resistant Salmonella isolates that had previously been isolated from cattle or the environment on seven dairy farms in New York State (36) in order to better understand the ecology and transmission of ceftiofur-resistant Salmonella.

**Materials and Methods**

**Salmonella isolates.** All isolates included in this study were obtained as part of a field study examining the effects of antimicrobial treatment on serogroup B Salmonella infections in New York dairy herds (36). All Salmonella isolates included in the present study were collected from cattle or the environment of seven farms which had at least one isolate with reduced susceptibility to ceftiofur. While these seven farms reported previous ceftiofur administration in cattle, so did 94% of farms in this field study. From the total number of Salmonella isolates collected on these farms, a subset of 39 isolates (supplemental Table S1, available at http://www.foodscience.cornell.edu/wiedmann/Alcaine%20Supplemental%20TSIpdf.pdf) was selected for further characterization. This subset contained isolates that were selected so that at least one isolate of each Salmonella serotype obtained on a given farm was included in our isolate set. For serotypes which included ceftiofur-resistant isolates, one or more resistant isolates as well as one or more sensitive isolates of a given serotype were selected, if sensitive isolates were available. All isolates were serotyped at the National Veterinary Services Laboratory (USDA Animal and Plant Health Inspection Service-Veterinary Services, Ames, IA).

**Antibiotic resistance profiles.** To characterize the antimicrobial isolates, Standard National Antimicrobial Resistance Monitoring System (34) panels were performed at the New York State Animal Health Diagnostic Center (Cornell University, Ithaca, NY) using the Sensititre system (Trek Diagnostic Systems Ltd., Cleveland, OH). Isolates were recovered from either lyophilized stocks or stocks stored using Microbank cryovials (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). The antimicrobial agents tested included amikacin, amoxicillin/clavulanic acid (Amc), ampicillin (Amp), cefoxitin (Fox), cefotaxin (Cef), ceftriaxone (Crx), chloramphenicol (Chl), ciprofloxacin, gentamicin (Gen), kanamycin (Kan), nalidixic acid, streptomycin (Str), sulfisoxazole (Suf), tetracycline (Tet), and trimethoprim/sulfamethoxazole (Smx). For ceftiofur and for streptomycin, antibiotic resistance results were not interpreted by the Sensititre system; the resistance cutoff for these antimicrobials was set at ≥ 8 μg/ml for ceftiofur and >32 μg/ml for streptomycin. The cutoff for ceftiofur has not been clinically validated, and therefore the classification of isolates for this study as ceftiofur resistant is not necessarily related to clinical efficacy.

**PCR and DNA sequencing.** Salmonella lysates for PCR were prepared by following a previously described protocol (13). PCR amplification was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA). PCR conditions and primer sequences for the amplification of the three genes (manB, fimA, and mdh) used for multilocus sequence typing (MLST) are presented in Table 1. MLST was performed essentially as previously described (33).

All PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc., Chatsworth, CA) and quantified using the fluorescent DNA quantitation kit (Bio-Rad, Hercules, CA). PCR products were sequenced at the Biotechnology Resource Center at Cornell University using the respective PCR primers (Table 1). All sequences were assembled and proofread using SeqMan and aligned using the Clustal W algorithm in MegAlign (DNASTar, Madison, WI).

PCR was also used to screen for the presence of the antibiotic resistance genes bla<sub>CMY-2</sub> and floR, using the conditions and primers listed in Table 1. For bla<sub>CMY-2</sub>-positive isolates, a full-length PCR amplicon was created using ampC primers (Table 1). This ampC amplicon was purified as described above and sequenced using both ampC and bla<sub>CMY-2</sub> forward and reverse primers (Table 1).

**MLST.** The MLST scheme used here was based on the sequencing of three genes, manB, fimA, and mdh, as previously reported (33). Allele assignments for manB and mdh were based on 640- and 520-bp sequence alignments, representing 47 and 55% of the respective open reading frames (ORFs). Allele assignments for fimA were based on a 558-bp sequence alignment, representing 100% of the ORF and 15 bp upstream of the fimA start codon. Allele assignments were performed using DnaSP 4.0 (31); two sequences were assigned different allelic types if they differed by at least 1 nucleotide. Allele assignments were performed to be consistent with allelic types previously reported by Sukhnanand et al. (33), e.g., allelic type 2 in this study is identical to allelic type 2 reported by Sukhnanand et al. (33).

**Evolutionary analyses.** Sukhnanand et al. (33) previously showed that a concatenated gene sequence of manB, fimA, and mdh showed limited evidence for reticulate evolution and thus concluded that meaningful phylogenetic trees could be constructed from a concatenated gene manB, fimA, and mdh sequence. We thus constructed a concatenated manB, fimA, and mdh for all 39 isolates included in this study. MODELTEST (27) was used to find the most likely model of DNA evolution for the concatenated sequence.
substitution for the concatenated sequence alignment, and PAUP* 4.0b10 (Si-
nauer Associates, Sunderland, MA) was used to construct maximum-likelihood
trees using the TrN + G substitution model, which was selected by MODELLTEST,
and 100 bootstrap replicates. The tree was rooted with a concatenated manB,
jfaA, and mdh sequence for Escherichia coli O157:H7 (16), which served as the
outgroup.

Access to detailed isolate information. All isolate information for this study,
such as isolate source, gene sequence data, and allele assignments, can be
accessed via the PathogenTracker website at www.pathogentracker.net; isolates
specifically included in the study reported here are listed at http://cbsusrv01.
tc.cornell.edu/users/PathogenTracker/pt2/search/display_list.aspx?refid=

RESULTS AND DISCUSSION

In order to better understand the mechanisms behind the transmission and spread of ceftiofur-resistant Salmonella in
dairy herds, an MLST scheme, as well as phenotypic and PCR-
based methods to detect the presence of selected antibiotic
resistance genes, was used for characterization of selected ceft-
iofur-resistant and -sensitive Salmonella isolates previously col-
lected from seven farms in New York State. MLST was chosen
as a typing method due to its ability to differentiate between
serotypes and provide information on the genetic relationship
between isolates (33). Our data indicate that (i) resistance to
ceftriaxone and ceftiofur is highly correlated with the presence of
blaCMY-2; (ii) ceftiofur-resistant Salmonella strains are geo-
graphically widespread, as shown by their isolation from farms
located throughout New York State; (iii) ceftiofur-resistant
Salmonella strains isolated from farms represent multiple dis-
tinct subtypes and evolutionary lineages, as determined by
serotyping, DNA sequence typing, and antimicrobial-resis-
tance profiles; and (iv) ceftiofur-resistant Salmonella evolved
by multiple independent acquisitions of an identical blaCMY-2
allele and by clonal spread of ceftiofur-resistant subtypes.

Resistance to ceftriaxone and ceftiofur is highly correlated
with the presence of blaCMY-2. Resistance to ceftiofur has been
linked to CMY-2, a plasmid-encoded AmpC-like beta-lacta-
mase (5, 38). All 19 ceftiofur-resistant isolates were found to
carry the gene blaCMY-2, and 17 of these isolates also showed at
least intermediate resistance to ceftriaxone, as defined by Sen-
sittre system analysis. There were no ceftiofur-sensitive iso-
lates that harbored blaCMY-2. In addition, 24 isolates harbored
floR, which encodes chloramphenicol resistance (37). All 19
of the ceftiofur-resistant isolates carried this gene, which is
consistent with previous studies that have found that floR
can sometimes be found on plasmids carrying blaCMY-2 (10).
Plasmids from the 19 ceftiofur-resistant isolates were typed
using the method described by Giles et al. (14). Of the 19
isolates, 15 were found to harbor type B plasmids, while the
remaining 4 did not carry plasmids that were typeable using
this method. Isolates carrying blaCMY-2 showed a range of
MICs for ceftriaxone (supplemental Table S1, available at http:
//www.foodscience.cornell.edu/wiedmann/Akcaine%20Supplemental%20TS1.pdf). Previous studies on
ampC-mediated antibiotic resistance in other Enterobacteriaceae
did not show a clear relationship between plasmid copy number and
resistance gene transcription and MICs (30), indicating that
elucidation of underlying mechanisms responsible for MIC dif-
fferences may be complicated.

The presence of blaCMY-2 was also associated with multiple-
drug resistance. All 19 isolates harboring blaCMY-2 showed
resistance to seven other antibiotics, including ampicillin,
bla\textsubscript{CMY-2} (Fig. 2). Evolutionary analysis of the 39 isolates revealed that they formed three strongly supported clades including one containing \textit{Salmonella} serotype Typhimurium and Typhimurium subsp. Copenhagen isolates (MLST types 6 and 8), one containing \textit{Salmonella} serotype Agona isolates (MLST types 1 and 2), and one containing \textit{Salmonella} serotype Schwarzengrund isolates (MLST type 4). The sole serotype Anatum isolate grouped close to the serotype Schwarzengrund clade, but its branch was not supported by a high bootstrap value (<50). Within the \textit{Salmonella} serotype Agona clade, there were two distinct lineages, one which contained all isolates that were \textit{bla}\textsubscript{CMY-2} positive and resistant to ceftiofur and one which only contained ceftiofur-sensitive isolates. While both lineages within the serotype Typhimurium/Typhimurium subsp. Copenhagen clade contained isolates that carried \textit{bla}\textsubscript{CMY-2}, neither the serotype Schwarzengrund nor the serotype Anatum isolates were resistant to ceftiofur.

\textbf{Ceftiofur-resistant \textit{Salmonella} evolved by independent emergence and clonal spread.} Our data suggest that both multiple independent acquisitions of \textit{bla}\textsubscript{CMY-2} and clonal spread of \textit{bla}\textsubscript{CMY-2} positive \textit{Salmonella} contribute to the distribution of

\begin{table}[h]
\centering
\begin{tabular}{lllll}
\hline
FSL designation & Serotype & Farm & MLST type & Resistance profile$^a$\\
\hline
FSL A4-021 & Agona & 36 & 1 & Sensitive to all \\
FSL A4-005 & Schwarzengrund & 36 & 4 & Sensitive to all \\
FSL S3-903 & Schwarzengrund & 36 & 4 & Sensitive to all \\
FSL S3-904 & Schwarzengrund & 36 & 4 & Sensitive to all \\
FSL A4-027 & Typhimurium & 36 & 4 & Sensitive to all \\
FSL A4-004 & Typhimurium & 36 & 6 & Sensitive to all \\
FSL S5-316 & Typhimurium subsp. Copenhagen & 36 & 6 & Sensitive to all \\
FSL A4-009 & Anatum & 77 & 1 & SulfTet \\
FSL S3-905 & Schwarzengrund & 100 & 4 & Sensitive to all \\
FSL A4-018 & Typhimurium & 111 & 6 & Sensitive to all \\
FSL S5-320 & Agona & 77 & 1 & SulfTet \\
FSL S5-325 & Typhimurium subsp. Copenhagen & 111 & 6 & ChlSulfStr \\
FSL A4-019 & Typhimurium subsp. Copenhagen & 111 & 6 & ChlSulfStrTet \\
FSL S5-324 & Typhimurium & 111 & 6 & AmpChlGenKanSulfStrTet \\
FSL A4-006 & Typhimurium & 46 & 6 & AmpAmc$^c$ChlKanSulfStrTet \\
FSL S5-318 & Typhimurium & 46 & 6 & AmpAmc$^c$ChlKanSulfStrTet \\
FSL A4-022 & Typhimurium subsp. Copenhagen & 100 & 8 & AmpKanSulfStrTet \\
FSL A4-025 & Typhimurium subsp. Copenhagen & 100 & 8 & AmpKanSulfStrTet \\
FSL A4-028 & Typhimurium subsp. Copenhagen & 100 & 8 & AmpKanSulfStrTet \\
FSL S5-322 & Typhimurium subsp. Copenhagen & 100 & 8 & AmpKanSulfStrTet \\
FSL S3-908 & Typhimurium & 111 & 6 & AmpAmcFoxCefGenKanSulfStrTet \\
FSL A4-014 & Typhimurium & 111 & 6 & AmpAmcFoxCefGenKanSulfStrTet \\
FSL A4-016 & Typhimurium & 36 & 6 & AmpAmcFoxCefCro$^c$GenKanSulfStrTet \\
FSL A4-017 & Typhimurium & 36 & 6 & AmpAmcFoxCefCro$^c$GenKanSulfStrTet \\
FSL A4-012 & Typhimurium & 111 & 6 & AmpAmcFoxCefCro$^c$GenKanSulfStrTet \\
FSL A4-013 & Typhimurium & 111 & 6 & AmpAmcFoxCefCro$^c$GenKanSulfStrTet \\
FSL A4-015 & Typhimurium & 111 & 6 & AmpAmcFoxCefCro$^c$GenKanSulfStrTet \\
FSL S5-315 & Typhimurium & 36 & 6 & AmpAmcFoxCefCroGenKanSulfStrTet \\
FSL A4-032 & Typhimurium & 36 & 6 & AmpAmcFoxCefCro$^c$ChlSulfStrTet \\
FSL A4-001 & Typhimurium & 14 & 8 & AmpAmcFoxCefCro$^c$ChlSulfStrTet \\
FSL A4-002 & Typhimurium & 14 & 8 & AmpAmcFoxCefCro$^c$ChlSulfStrTet \\
FSL A4-003 & Typhimurium subsp. Copenhagen & 14 & 8 & AmpAmcFoxCefCro$^c$ChlSulfStrTet \\
FSL S3-913 & Typhimurium subsp. Copenhagen & 14 & 8 & AmpAmcFoxCefCro$^c$ChlSulfStrTet \\
FSL S3-911 & Typhimurium subsp. Copenhagen & 100 & 8 & AmpAmcFoxCefCro$^c$ChlSulfStrTet \\
FSL S3-900 & Agona & 38 & 2 & AmpAmcFoxCefCro$^c$ChlKanSulfStrTetSxt \\
FSL S5-317 & Agona & 38 & 2 & AmpAmcFoxCefCro$^c$ChlKanSulfStrTetSxt \\
FSL A4-007 & Agona & 46 & 2 & AmpAmcFoxCefCro$^c$ChlKanSulfStrTetSxt \\
FSL S5-319 & Agona & 46 & 2 & AmpAmcFoxCefCro$^c$ChlKanSulfStrTetSxt \\
FSL A4-008 & Agona & 77 & 2 & AmpAmcFoxCefCro$^c$ChlKanSulfStrTetSxt \\
\hline
\end{tabular}
\caption{Serotype, MLST, and antibiotic resistance profiles of \textit{Salmonella} isolates}
\end{table}

$^a$ Intermediate resistance, e.g., Cro$^c$ indicates intermediate resistance to ceftriaxone.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Distribution of MLST types across New York dairy farms. Numbers in parenthesis indicate farm number, and boldface numbers indicate MLST types isolated on the respective farms.}
\end{figure}
ceftiofur-resistant *Salmonella*. Sequencing of *bla*<sub>CMY-2</sub> revealed that all isolates carried an identical allele, suggesting that the gene was acquired from a common source. The presence of an identical *bla*<sub>CMY-2</sub> allele in three MLST types representing distinct evolutionary lineages in geographically dispersed farms suggests multiple, independent acquisitions of this gene. From our data, we could not determine the primary source of *bla*<sub>CMY-2</sub> but other research has shown that the gene is carried in several enterobacteria and that the transfer of plasmids containing *bla*<sub>CMY-2</sub> between these organisms does occur (39, 40). Further research is needed to determine whether *bla*<sub>CMY-2</sub> is transferred between *Salmonella* or whether it has been acquired multiple times from another bacterial species.

Evidence for clonal spread of *bla*<sub>CMY-2</sub>-positive *Salmonella* is provided by isolates obtained from farms 46, 38, and 77. Specifically, MLST type 2 *Salmonella* serotype Agona isolates carrying an identical *bla*<sub>CMY-2</sub> allele and displaying identical antibiotic resistance profiles were isolated from each of these farms and represented the only ceftiofur-resistant *Salmonella* strains isolated on these farms. The high level of genotypic and

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**TABLE 3. Allelic profiles and MLST types of *Salmonella* isolates**

<table>
<thead>
<tr>
<th>Serotype (no. of isolates)</th>
<th>Allelic profile&lt;sup&gt;a&lt;/sup&gt; for:</th>
<th>MLST type&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fimA</td>
<td>mdh</td>
</tr>
<tr>
<td>Agona (2)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Agona (5)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Schwarzengrund (5)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Typhimurium (14)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Typhimurium subsp. Copenhagen (3)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Typhimurium (2)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Typhimurium subsp. Copenhagen (7)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Anatum (1)</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> MLST and allelic types were assigned to be consistent with Sukhnanand et al. (32).

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**FIG. 2. Phylogenetic tree of *Salmonella* isolates based on the concatenated *manB*, *mdh*, and *fimA* sequences.** The phylogenetic tree was built using the maximum-likelihood method and the TrN+G model, which was selected by MODELTEST as the best model. The outgroup branch length was collapsed for easier viewing. Bootstrap values >50.0 are indicated at the node of the branch. Numbers in parentheses indicate farm numbers, C indicates the presence of *bla*<sub>CMY-2</sub>, and F indicates the presence of *floR*. The scale bar indicates relative sequence distance.
phenotypic similarity between these isolates suggests that they belong to a clonal group whose evolutionary ancestor acquired blaCMY-2 and spread, at least, across New York State. A Salmonella serotype Agona isolate with a very similar antibiotic resistance profile was isolated from turkey meat in the Washington, D.C., area (7), suggesting that this clonal group may be present in other U.S. regions. The fact that this serotype Agona subtype is easily identifiable via an MLST scheme suggests that MLST monitoring of Salmonella may provide a rapid and accurate method for the identification of this multidrug-resistant subtype.

Further evidence of independent emergence followed by clonal spread was found through Salmonella isolated on farms 14 and 100. On both these farms, MLST type 8 isolates harboring blaCMY-2 and displaying identical antibiotic resistance profiles were identified. All isolates from farm 14 appeared to be part of this clonal group, whereas only one isolate from farm 100 was classified into this clonal group (Table 2). Other serotype Typhimurium isolates displaying similar antibiotic resistance profiles have been isolated from humans in Ohio and California (5), but the lack of genetic information on these Salmonella subtypes makes it difficult to compare data across studies and to define the spread and distribution of these new subtypes. Use of an MLST monitoring scheme for Salmonella would provide a standardized method to analyze clinical isolates and rapidly identify emerging antibiotic-resistant clonal groups.

In summary, blaCMY-2, which encodes ceftiofur/ceftriaxone resistance, appeared to be present on a highly mobile genetic element that was readily acquired. Following blaCMY-2 acquisition, ceftiofur-resistant Salmonella subtypes may spread widely. These subtypes also seem to often display multidrug resistance and, without proper identification and treatment, may present a serious human health risk (3, 17). Continued monitoring will be necessary to detect the emergence and spread of cephapolin-resistant Salmonella through animal and human populations.

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