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* subtypes, including the appearance of subtypes resistant to ceftriaxone, represents a particular concern. Ceftriaxone is used to treat invasive cases of *Salmonella* in children and is closely related to ceftiofur, an antibiotic commonly used to treat diseases of cattle. In order to develop a better understanding of the evolution and transmission of ceftiofur resistance in *Salmonella*, we characterized ceftiofur-resistant and -sensitive *Salmonella* isolates from seven New York dairy farms. A total of 39 isolates from these seven farms were analyzed for evolutionary relatedness (by DNA sequencing of the *Salmonella* genes fimA, manB, and mdh), antibiotic resistance profiles, and the presence of *blaCMY-2*, a beta-lactamase gene associated with resistance to cephaporsins. Our data indicate that (i) resistance to ceftriaxone and ceftiofur was highly correlated with the presence of *blaCMY-2*; (ii) ceftiofur-resistant *Salmonella* strains were geographically widespread, as shown by their isolation from farms located throughout New York State; (iii) ceftiofur-resistant *Salmonella* strains isolated from farms represent multiple distinct subtypes and evolutionary lineages, as determined by serotyping, DNA sequence typing, and antimicrobial-resistance profiles; and (iv) ceftiofur-resistant *Salmonella* strains evolved by multiple independent acquisitions of an identical *blaCMY-2* allele and by clonal spread of ceftiofur-resistant subtypes.

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 ceftriazone-resistant *Salmonella* infection in a child (12, 32), further data on the transmission and evolution of ceftiofur- and ceftriazone-resistant *Salmonella* strains are needed.

The most common mechanism of cephalosporin resistance is the production of beta-lactamases. Cephalosporins are semisynthetic antibiotics originally derived from cephalosporin C, a naturally occurring antimicrobial produced by *Cephalosporium acremonium*. Like other beta-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 beta-lactam ring. Beta-lactamases confer resistance by hydrolyzing the beta-lactam ring, producing beta-amino acids with no antimicrobial activity (20). Broad-spectrum cephalosporins,
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 ceftriaxone and cefotaxime 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 blaCMY-2 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 blaCMY-2 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 ceftriaxone-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 ceftriaxone-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 ceftriaxone. While these seven farms reported previous ceftriaxone 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/Aalcaine%20Supplemental%20Table1.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 ceftriaxone-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 resistance of the isolates, Standard National Antimicrobial Resistance Monitoring System (SNARMS) panels were performed at the New York State Animal Health Diagnostic Center (Cornell University, Ithaca, NY) using the Sensititre system (Trekar 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), cefotaxim (Cef), ceftriaxone (Cra), chloramphenicol (Chi), ciprofloxacin, gentamicin (Gen), kanamycin (Kan), nalidixic acid, streptomycin (Str), sulfisoxazole (Sulf), tetracycline (Tet), and trimethoprim/sulfamethoxazole (Sxt). For ceftriaxone 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 ceftriaxone and >32 µg/ml for streptomycin. The cutoff for ceftriaxone has not been clinically validated, and therefore the classification of isolates for this study as ceftriaxone 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 AmpliTag 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 quantification system; the resistance cutoff for these antimicrobials was set at 50% for ceftriaxone 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 ceftriaxone and >32 µg/ml for streptomycin. The cutoff for ceftriaxone has not been clinically validated, and therefore the classification of isolates for this study as ceftriaxone resistant is not necessarily related to clinical efficacy.

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**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 this concatenated dataset.

**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 alignment, 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).
substitution for the concatenated sequence alignment, and PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) was used to construct maximum-likelihood trees using the TrN+G substitution model, which was selected by MODELTEST, and 100 bootstrap replicates. The tree was rooted with a concatenated mabB, fimA, 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 http://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=241.

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 ceftiofur-resistant and -sensitive Salmonella isolates previously collected 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 \( \text{bla}_{\text{CMY-2}} \); (ii) ceftiofur-resistant Salmonella strains are geographically widespread, as shown by their isolation from farms located throughout New York State; (iii) ceftiofur-resistant Salmonella strains isolated from farms represent multiple distinct subtypes and evolutionary lineages, as determined by serotyping, DNA sequence typing, and antimicrobial-resistance profiles; and (iv) ceftiofur-resistant Salmonella evolved by multiple independent acquisitions of an identical \( \text{bla}_{\text{CMY-2}} \) allele and by clonal spread of ceftiofur-resistant subtypes.

Resistance to ceftriaxone and ceftiofur is highly correlated with the presence of \( \text{bla}_{\text{CMY-2}} \). Resistance to ceftiofur has been linked to CMY-2, a plasmid-encoded AmpC-like beta-lactamase (5, 38). All 19 ceftiofur-resistant isolates were found to carry the gene \( \text{bla}_{\text{CMY-2}} \), and 17 of these isolates also showed at least intermediate resistance to ceftriaxone, as defined by Sensititre system analysis. There were no ceftiofur-resistant isolates that harbored \( \text{bla}_{\text{CMY-2}} \). In addition, 24 isolates harbored \( \text{floR} \), 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 \( \text{floR} \) can sometimes be found on plasmids carrying \( \text{bla}_{\text{CMY-2}} \) (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 \( \text{bla}_{\text{CMY-2}} \) showed a range of MICs for ceftriaxone (supplemental Table S1, available at http://www.foodscience.cornell.edu/wiedmann/Akaine%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 differences may be complicated.

The presence of \( \text{bla}_{\text{CMY-2}} \) was also associated with multiple-drug resistance. All 19 isolates harboring \( \text{bla}_{\text{CMY-2}} \) showed resistance to seven other antibiotics, including amoxicillin, amoxacillin, cefoxitin, chloramphenicol, sulfisoxazole, streptomycin, and tetracycline (Table 2). In addition, 18 of these isolates also showed resistance to kanamycin, and all five ceftiofur-resistant isolates of Salmonella enterica serotype Agona showed resistance to trimethoprim-sulfamethoxazole. Similar antibiotic resistance patterns have been noted in other studies (5, 7). Specifically, Carattoli et al. (5) found a resistance profile similar to that found in our MLST type 2 Salmonella serotype Agona in a human S. enterica serotype Typhimurium isolate from Oregon, and Chen et al. (7) also reported similar resistance profile in Salmonella serotype Agona isolates obtained from ground turkey in the United States. Carattoli et al. (5) also found a human Salmonella serotype Typhimurium isolate from New York State and a human S. enterica serotype Newport isolate from Kansas with resistance profiles similar to a MLST type 6 Salmonella serotype Typhimurium isolate reported here.

Multiple-drug resistance was not as common in isolates lacking \( \text{bla}_{\text{CMY-2}} \). One-half of the isolates lacking \( \text{bla}_{\text{CMY-2}} \) were sensitive to all antimicrobials tested, and three showed resistance to three or fewer of the antimicrobials tested. The remaining seven isolates showed resistance to ampicillin, kanamycin, sulfisoxazole, streptomycin, and tetracycline. Of these isolates, two also showed resistance to chloramphenicol and intermediate resistance to amoxicillin and one showed resistance to chloramphenicol and gentamicin.

Ceftiofur-resistant Salmonella strains are geographically widespread. Ceftiofur-resistant Salmonella strains were isolated from farms across New York State (Fig. 1) (36). Salmonella strains harboring \( \text{bla}_{\text{CMY-2}} \) have also been previously isolated from cattle in Iowa and Pennsylvania (29, 38); humans in California, Colorado, Nebraska, Oregon, Kansas, and Massachusetts (5); and retail meats in the Washington, D.C., metropolitan area (7). In addition, an outbreak of Salmonella serotype Newport, which was resistant to ceftiofur, in humans was reported in 2002 in five states including New York, Michigan, Pennsylvania, Ohio, and Connecticut (41). Ceftiofur-resistant Salmonella strains thus appear to be widespread within the United States and may pose a growing problem for effective antibiotic treatment of Salmonella infections (15).

Ceftiofur-resistant Salmonella strains represent multiple distinct subtypes and evolutionary lineages. MLST grouped the 39 isolates tested into six distinct MLST types, encompassing five different serotypes (Table 3). Serotypes Schwarzengrund and Anatum each represented a single MLST type, whereas serotype Agona could be differentiated into two MLST types. MLST types 8 and 6 contained both Typhimurium and Typhimurium subsp. Copenhagen serotypes. The difficulty in differentiating these two serotypes with an MLST scheme was expected due to their high genetic similarity (28). Of the six MLST types, only MLST type 2 serotype Agona, MLST type 6 serotype Typhimurium, and MLST type 8 serotype Typhimurium subsp. Copenhagen contained isolates with ceftiofur resistance. While these serotypes have previously been found among ceftiofur-resistant Salmonella strains isolated from cattle, humans, and retail meats (5, 7, 38), other serotypes found as harboring \( \text{bla}_{\text{CMY-2}} \) included Newport, Infantis, and Seftenberg (7, 19, 29, 41).

Our data showed that, within the five serotypes found in this study, there were distinct evolutionary lineages that harbor
Evolutionary analysis of the 39 isolates revealed that they formed three strongly supported clades including one containing *Salmonella* serotype Typhimurium and Typhimurium subsp. Copenhagen isolates (MLST types 6 and 8), one containing *Salmonella* serotype Agona isolates (MLST types 1 and 2), and one containing *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 *Salmonella* serotype Agona clade, there were two distinct lineages, one which contained all isolates that were bla<sub>CMY-2</sub> 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 bla<sub>CMY-2</sub>, neither the serotype Schwarzengrund nor the serotype Anatum isolates were resistant to ceftiofur.

Ceftiofur-resistant *Salmonella* evolved by independent emergence and clonal spread. Our data suggest that both multiple independent acquisitions of bla<sub>CMY-2</sub> and clonal spread of bla<sub>CMY-2</sub> positive *Salmonella* contribute to the distribution of bla<sub>CMY-2</sub> (Fig. 2).
ceftiofur-resistant Salmonella. Sequencing of bla\textsubscript{CMY-2} revealed that all isolates carried an identical allele, suggesting that the gene was acquired from a common source. The presence of an identical bla\textsubscript{CMY-2} 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\textsubscript{CMY-2} but other research has shown that the gene is carried in several enterobacteria and that the transfer of plasmids containing bla\textsubscript{CMY-2} between these organisms does occur (39, 40). Further research is needed to determine whether bla\textsubscript{CMY-2} is transferred between Salmonella or whether it has been acquired multiple times from another bacterial species.

Evidence for clonal spread of bla\textsubscript{CMY-2}-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\textsubscript{CMY-2} 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

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

<table>
<thead>
<tr>
<th>Serotype (no. of isolates)</th>
<th>Allelic profile(^a) for:</th>
<th>MLST type(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agona (2)</td>
<td>1 2 1 1</td>
<td>1</td>
</tr>
<tr>
<td>Agona (5)</td>
<td>1 1 2 2</td>
<td>2</td>
</tr>
<tr>
<td>Schwarzengrund (5)</td>
<td>3 4 4 4</td>
<td>4</td>
</tr>
<tr>
<td>Typhimurium (14)</td>
<td>4 5 5 6</td>
<td>6</td>
</tr>
<tr>
<td>Typhimurium subsp. Copenhagen (3)</td>
<td>4 5 5 6</td>
<td>6</td>
</tr>
<tr>
<td>Typhimurium (2)</td>
<td>4 5 7 8</td>
<td>8</td>
</tr>
<tr>
<td>Typhimurium subsp. Copenhagen (7)</td>
<td>4 5 7 8</td>
<td>8</td>
</tr>
<tr>
<td>Anatum (1)</td>
<td>6 12 18 25</td>
<td>25</td>
</tr>
</tbody>
</table>

\(^a\) MLST and allelic types were assigned to be consistent with Sukhnanand et al. (32).

**FIG. 2.** Phylogenetic tree of Salmonella isolates based on the concatenated \textit{manB}, \textit{mdh}, and \textit{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 \textit{bla}\textsubscript{CMY-2}, and F indicates the presence of \textit{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 bla\textsubscript{CMY-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 isolate with a very similar antibiotic resistance, appeared to be present on a highly mobile genetic background would provide a standardized method to analyze clinical isolates and rapidly identify emerging antibiotic-resistant clonal groups.

In summary, bla\textsubscript{CMY-2}, which encodes ceftriaxone resistance, appeared to be present on a highly mobile genetic element that was readily acquired. Following bla\textsubscript{CMY-2} acquisition, ceftriaxone-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 cephalosporin-resistant Salmonella through animal and human populations.

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REFERENCES