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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 bla_{CMY-2}, a beta-lactamase gene associated with resistance to cephalosporins. Our data indicate that (i) resistance to ceftriaxone and ceftiofur was highly correlated with the presence of bla_{CMY-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 bla_{CMY-2} allele and by clonal spread of ceftiofur-resistant subtypes.**

Salmonella is a gram-negative, rod-shaped bacillus that lives in the intestines of mammals, birds, and reptiles. It is shed into the environment in the feces of infected hosts and can survive in water, soil, and food for extended periods of time (2). Most human *Salmonella* infections in developed countries are acquired through consumption of contaminated food or contact with infected animals. In the United States, *Salmonella* is the second most common identifiable cause of illness, and the leading cause of hospitalizations and deaths, due to food-borne bacterial infection (21). While most *Salmonella* infections result in temporary gastroenteritis that usually does not require treatment (23), invasive *Salmonella* infections generally require antimicrobial treatment (4, 35). Traditionally, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole were used to treat such severe cases, but the increasing number 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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TABLE 1. PCR primers and conditions

Gene	Primers (forward, reverse)	PCR conditions[a]	Reference
manB	5′-CAT AAY CCG ATG GAC TAC AAC G-3′, 5′-ACC AGC AGC CAC GGG ATC AT-3′	95°C/9.5 min (1 cycle); 95°C/45 s, TD 55–45°C/45 s, 72°C/60 s (40 cycles); 72°C/7 min (1 cycle)	32
mdh	5′-GAT GAA AGT CGC AGT CCT CG-3′, 5′-TAT CCA GCA TAG CGT CCA GC-3′	95°C/9.5 min (1 cycle); 95°C/45 s, TD 58–48°C/45 s, 72°C/60 s (40 cycles), 72°C/7 min (1 cycle)	32
fimA	5′-TCA GGG GAG AAA CAG AAA ACT AAT-3′, 5′-TCC CCG ATA GCC TCT TCC-3′	95°C/9.5 min (1 cycle); 95°C/45 s, 57°C/45 s, 72°C/60 s (35 cycles); 72°C for 7 min (1 cycle)	32
bla_{CMY-2}	5′-TGG CCA GAA CTG ACA GGC AAA-3′, 5′-TTT CTC CTG AAC GTG GCT GGC-3′	95°C/9.5 min (1 cycle); 95°C/45 s, 60°C/45 s, 72°C/60 s (40 cycles); 72°C/7 min (1 cycle)	26
ampC	5′-AAC ACA CTG ATT GCG TCT GAC-3′, 5′-CTG GGC CTC ATC GTC AGT TA-3′	95°C/9.5 min (1 cycle); 95°C/45 s, 60°C/45 s, 72°C/60 s (40 cycles); 72°C/7 min (1 cycle)	26
floR	5′-CTG AGG GTG TCG TCA TCT AC-3′, 5′-GCT CCG ACA ATG CTG ACT AT-3′	95°C/9.5 min (1 cycle); 95°C/30 s, 55°C/60 s, 72°C/60 s (40 cycles); 72°C/7 min (1 cycle)	7

[a] TD, touchdown PCR; over the first 20 cycles annealing temperature is decreased by 0.5°C/cycle, followed by 20 cycles at the lower annealing temperature (i.e., temperature reached after the last TD cycle).

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_{CMY-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 bla_{CMY-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 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%20TS1.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 resistance of the 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), ceftiotur (Cef), ceftriaxone (Cro), chloramphenicol (Chl), ciprofloxacin, gentamicin (Gen), kanamycin (Kan), nalidixic acid, streptomycin (Str), sulfisoxazole (Suf), tetracycline (Tet), and trimethoprim/sulfamethoxazole (Sxt). 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 Ampli*Taq* 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 by 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_{CMY-2} and *floR*, using the conditions and primers listed in Table 1. For bla_{CMY-2}-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_{CMY-2} 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

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 *manB*, *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 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=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 bla_{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 bla_{CMY-2} allele and by clonal spread of ceftiofur-resistant subtypes.

Resistance to ceftriaxone and ceftiofur is highly correlated with the presence of bla_{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 bla_{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-sensitive isolates that harbored bla_{CMY-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 bla_{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 bla_{CMY-2} showed a range of MICs for ceftriaxone (supplemental Table S1, available at http://www.foodscience.cornell.edu/wiedmann/Alcaine%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 bla_{CMY-2} was also associated with multiple-drug resistance. All 19 isolates harboring bla_{CMY-2} showed resistance to seven other antibiotics, including ampicillin, amoxicillin, 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 bla_{CMY-2}. One-half of the isolates lacking bla_{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 bla_{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, a 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 bla_{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

TABLE 2. Serotype, MLST, and antibiotic resistance profiles of *Salmonella* isolates

FSL designation	Serotype	Farm	MLST type	Resistance profile[a]
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	Schwarzengrund	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	25	Sensitive to all
FSL S3-905	Schwarzengrund	100	4	Sensitive to all
FSL A4-018	Typhimurium	111	6	Sensitive to all
FSL S5-320	Agona	77	1	SufTet
FSL S5-325	Typhimurium subsp. Copenhagen	111	6	ChlSufStr
FSL A4-019	Typhimurium subsp. Copenhagen	111	6	ChlSufStrTet
FSL S5-324	Typhimurium	111	6	AmpChlGenKanSufStrTet
FSL A4-006	Typhimurium	46	6	AmpAmc*ChlKanSufStrTet
FSL S5-318	Typhimurium	46	6	AmpAmc*ChlKanSufStrTet
FSL A4-022	Typhimurium subsp. Copenhagen	100	8	AmpKanSufStrTet
FSL A4-025	Typhimurium subsp. Copenhagen	100	8	AmpKanSufStrTet
FSL A4-028	Typhimurium subsp. Copenhagen	100	8	AmpKanSufStrTet
FSL S5-322	Typhimurium subsp. Copenhagen	100	8	AmpKanSufStrTet
FSL S3-908	Typhimurium	111	6	AmpAmcFoxCefGenKanSufStrTet
FSL A4-014	Typhimurium	111	6	AmpAmcFoxCefGenKanSufStrTet
FSL A4-016	Typhimurium	36	6	AmpAmcFoxCefCro*GenKanSufStrTet
FSL A4-017	Typhimurium	36	6	AmpAmcFoxCefCro*GenKanSufStrTet
FSL A4-012	Typhimurium	111	6	AmpAmcFoxCefCro*GenKanSufStrTet
FSL A4-013	Typhimurium	111	6	AmpAmcFoxCefCro*GenKanSufStrTet
FSL A4-015	Typhimurium	111	6	AmpAmcFoxCefCro*GenKanSufStrTet
FSL S5-315	Typhimurium	36	6	AmpAmcFoxCefCroGenKanSufStrTet
FSL A4-032	Typhimurium	36	6	AmpAmcFoxCefCro*ChlSufStrTet
FSL A4-001	Typhimurium	14	8	AmpAmcFoxCefCro*ChlKanSufStrTet
FSL A4-002	Typhimurium	14	8	AmpAmcFoxCefCro*ChlKanSufStrTet
FSL A4-003	Typhimurium subsp. Copenhagen	14	8	AmpAmcFoxCefCro*ChlKanSufStrTet
FSL S3-913	Typhimurium subsp. Copenhagen	14	8	AmpAmcFoxCefCro*ChlKanSufStrTet
FSL S3-911	Typhimurium subsp. Copenhagen	100	8	AmpAmcFoxCefCro*ChlKanSufStrTet
FSL S3-900	Agona	38	2	AmpAmcFoxCefCro*ChlKanSufStrTetSxt
FSL S5-317	Agona	38	2	AmpAmcFoxCefCro*ChlKanSufStrTetSxt
FSL A4-007	Agona	46	2	AmpAmcFoxCefCro*ChlKanSufStrTetSxt
FSL S5-319	Agona	46	2	AmpAmcFoxCefCro*ChlKanSufStrTetSxt
FSL A4-008	Agona	77	2	AmpAmcFoxCefCro*ChlKanSufStrTetSxt

[a] *, intermediate resistance, e.g., Cro* indicates intermediate resistance to ceftriaxone.

bla_{CMY-2} (Fig. 2). 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_{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 bla_{CMY-2}, 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_{CMY-2} and clonal spread of bla_{CMY-2} positive *Salmonella* contribute to the distribution of

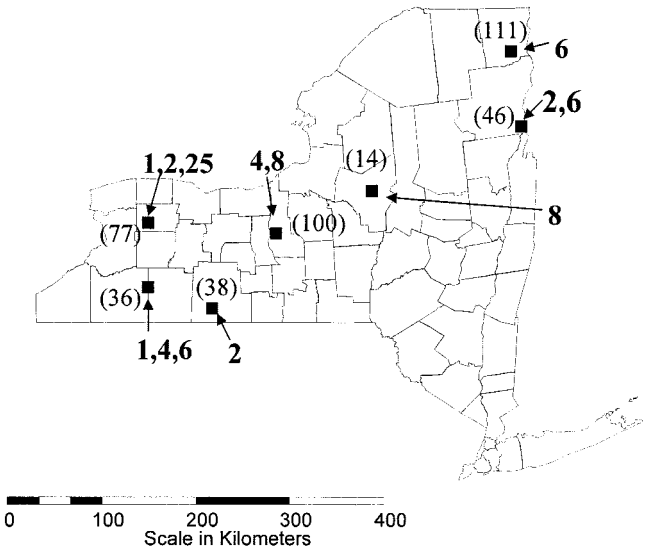

FIG. 1. Distribution of MLST types across New York dairy farms. Numbers in parenthesis indicate farm number, and boldface numbers indicate MLST types isolated on the respective farms.

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

Serotype (no. of isolates)	Allelic profile[a] for:			MLST type[a]
	fimA	*mdh*	*manB*	
Agona (2)	1	2	1	1
Agona (5)	1	1	2	2
Schwarzengrund (5)	3	4	4	4
Typhimurium (14)	4	5	5	6
Typhimurium subsp. Copenhagen (3)	4	5	5	6
Typhimurium (2)	4	5	7	8
Typhimurium subsp. Copenhagen (7)	4	5	7	8
Anatum (1)	6	12	18	25

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

ceftiofur-resistant *Salmonella*. Sequencing of bla_{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_{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_{CMY-2} but other research has shown that the gene is carried in several enterobacteria and that the transfer of plasmids containing bla_{CMY-2} between these organisms does occur (39, 40). Further research is needed to determine whether bla_{CMY-2} is transferred between *Salmonella* or whether it has been acquired multiple times from another bacterial species.

Evidence for clonal spread of bla_{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_{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

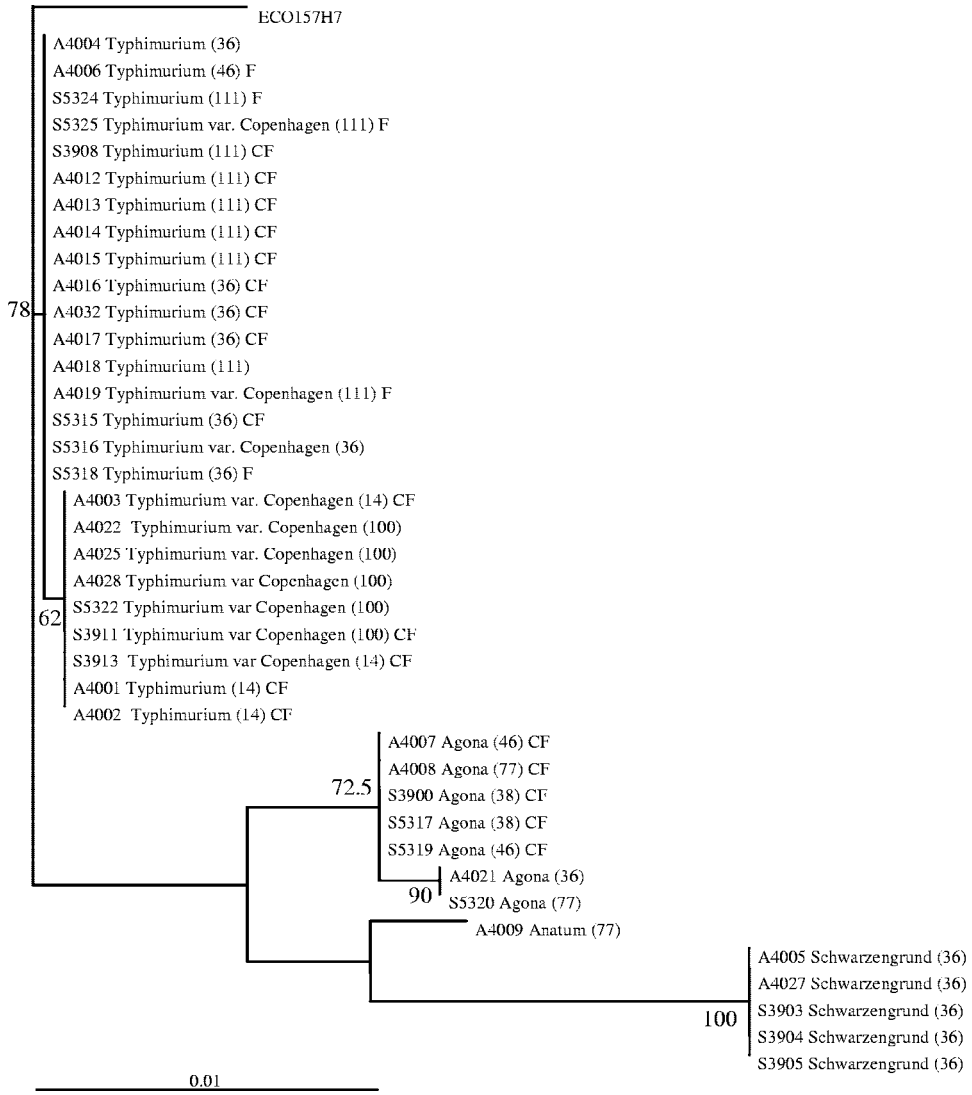

FIG. 2. Phylogenetic tree of *Salmonella* isolates based on the concatenated *manB*, *mdh*, and *fimA* sequences. The phylogenetic tree was built using the maximum-likelihood method and the TrN+G model, which was selected by MODELTEST as the best model. The outgroup branch length was collapsed for easier 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_{CMY-2}, 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 bla_{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 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 bla_{CMY-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, bla_{CMY-2}, which encodes ceftiofur/ceftriaxone resistance, appeared to be present on a highly mobile genetic element that was readily acquired. Following bla_{CMY-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 cephalosporin-resistant *Salmonella* through animal and human populations.

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