ANTIOBIOTIC RESISTANCE TRANMISSION AND MOLECULAR ECOLOGY OF *SALMONELLA ENTERICA* SUBTYPES FROM NEW YORK STATE

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ABSTRACT

Salmonella enterica is a gram-negative, rod shaped bacillus, which inhabits the intestines of mammals, reptiles and birds. In the United States, *Salmonella* is one of the leading causes of food-borne illness, and is typically acquired through the consumption of contaminated food or water. Modern public health practises have lead to a decrease in the number of *Salmonella* infections, however, there has been rise in the number of infections caused by antimicrobial-resistant *Salmonella.*

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 $bla_{\text{CMY-2}}$, a beta-lactamase gene associated with resistance to cephalosporins. Our data indicate that (i) resistance to ceftriaxone and ceftiofur were highly correlated with the presence of $bla_{\text{CMY-2}}$; (ii) ceftiofur resistant *Salmonella* were geographically widespread as shown by their isolation from farms located throughout New York state; (iii) ceftiofur resistant *Salmonella* 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 $bla_{\text{CMY-2}}$ allele and by clonal spread of ceftiofur resistant subtypes.

A collection of 179 human and 166 bovine clinical *Salmonella* isolates obtained from across New York State over the course of one year were characterized using serotyping and multilocus sequence typing (MLST) scheme based on the sequencing of three genes (*fimA*, *manB*, and *mdh*). The 345 isolates were differentiated into 52 serotypes and 75 sequence types (STs). Serotypes and STs were not randomly distributed among human and bovine isolates and selected serotypes and STs were exclusively associated with human and bovine isolates. A number of common STs were geographically widely distributed, including isolates representing the emerging *Salmonella* serotype 4,5,12:i:-, which was found among human and bovine isolates in a number of counties in New York state. Phylogenetic analyses supported that serotype 4,5,12:i:- is closely related to *Salmonella* Typhimurium and that *Salmonella* Newport represents two distinct evolutionary lineages that differ in their frequency in human and bovine isolates. A number of isolates carried two copies of *manB* (48 isolates) or showed small deletion events in *fimA* (9 isolates). Our data indicate that (i) serotyping and MLST typing both provide for sensitive subtype discrimination of *Salmonella*; (ii) bovine and human *Salmonella* subtypes represent distinct and overlapping populations; (iii) a number of *Salmonella* clonal groups, including emerging subtype 4,5,12:i:-, are geographically widespread among human and/or bovine populations; (iv) *Salmonella* Newport represents two distinct phylogenetic lineages that appear to be host specific; and (v) duplication and deletion events in *manB* and *fimA* may provide a mechanism for rapid diversification of *Salmonella* surface molecules.

BIOGRAPHICAL SKETCH

Samuel Alcaine was born on a cold Thanksgiving Day in 1979. He is considered a hero by some, a legend by others, and completely unknown to the majority of the world. Exactly why he was selected by Dr. Martin Wiedmann to join the Food Science Lab at Cornell University, he will never know. What he does know is that his time in Ithaca and the Wiedmann Lab was one of the most important experiences in his life, and the people he met there have shaped him for the better, or so he hopes. His greatest triumphs were achieved either while chilling with the members of I-ETA-PI at the hip and exclusive bar known as Korova, or while streaking plates and singing La Ley late at night in the Wiedmann lab. While those moments arguably changed the world we live in, they are not recorded in the following pages. What follows is Samuel Alcaine's attempt, with much help and prodding from Dr. Martin Wiedmann, to do something useful for society.

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CHAPTER 1

ANTIMOCROBIAL RESISTANCE IN *SALMONELLA*: A REVIEW*

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ABSTRACT

Salmonella is of one the leading causes of food-borne illness in countries around the world. Treatment of *Salmonella* infections, in both animals and humans, has become more difficult with the increased prevalence of *Salmonella* strains exhibiting multi-drug resistance. In order to implement methods to monitor and control the spread of multi-drug resistant (MDR) *Salmonella*, it is important to first understand the mechanisms responsible for drug-resistance and how drug-resistance is transmitted to and between *Salmonella* strains. This review will summarise what is currently known about multi-drug resistant *Salmonella*, the antimicrobials commonly used to treat *Salmonella* infections, the genes implicated in *Salmonella* drug-resistance, and the mechanisms responsible for the transmission of drug-resistance in *Salmonella*.

INTRODUCTION

Salmonella is a gram-negative rod shaped bacillus that causes infections in a large number of birds, mammals, and reptiles. The genus *Salmonella* includes 2 species, *Salmonella bongori* and *Salmonella enterica*, which is divided into 6 subspecies: I, II, IIIa, IIIb, IV, and VI (25). Subspecies I represents roughly 99% of all reported human isolates in the US (25). *Salmonella* isolates are traditionally classified via serotypes, which are based on the immunoreactivity of the O and H antigens. Over 2500 *Salmonella* serotypes have been identified, with the top 10 ten serotypes representing 74% of all *Salmonella* isolates reported in the US (25).

Salmonella infections are typically contracted through the consumption of contaminated food, feed or water or through direct contact with an infected host. Specifically, *Salmonella* is one of the leading causes of food-borne illness in the United States and the Europe Union (36, 69), where it is represents an incidence of 15.1 cases per 100,000 persons in the US (8), and 42.2 cases per 100,000 persons in

the EU (36). Most *Salmonella* infections do not require treatment, and result in temporary gastroenteritis (76). In more invasive, life-threatening cases, the use of antimicrobials is required (98), but the efficacy of many of theses drugs is decreasing as more antimicrobial resistant *Salmonella* subtypes emerge (5, 98, 106). The emergence and spread of antimicrobial resistant *Salmonella* strains, particularly those that are resistant to multiple antimicrobials (i.e., multi-drug resistant [MDR] *Salmonella*) is thus a major public health concern. The goal of this review is to provide an overview of the importance of the emergence of MDR *Salmonella*, with a focus on the mechanisms of drug resistance, as well as the occurrence and the transmission of drug resistance genes among members of the genus *Salmonella*.

HUMAN *SALMONELLA* INFECTIONS WITH DRUG-RESISTANT STRAINS

The rise of multi-drug resistant *Salmonella* poses an increased health risk to human populations (98). Of the top ten CDC reported human *Salmonella* serotypes (25), eight were found to include some strains that showed resistance to 5 or more antimicrobial agents (24). Serotypes Typhimurium, Heidelberg, and Newport most commonly exhibited multi-drug resistance (24). MDR Typhimurium isolates commonly showed two resistance type: (i) resistance to ampicillin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline (AKSSuT); or (ii) resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (ACSSuT), which is the resistance type typically associated with Typhimurium DT104 (24). The most common resistance type among Heidelberg isolates displayed resistance to ampicillin, amoxicillin-clavulanic acid, ceftiofur, and cephalothin (24). Among Newport isolates, the most commonly encountered resistance type is referred to as MDR-AmpC, which indicates resistance to ampicillin, chloramphenicol, amoxicillin-clavulanic acid, ceftiofur, cephalothin, streptomycin, sulfamethoxazole,

tetracycline, and reduced susceptibility to ceftriaxone (24). From 1993 to 2003, the CDC reported a 160% increase in the number of *Salmonella* serotype Newport isolates received (25). In 2002, 22% of Newport isolates tested by the National Antimicrobial Resistance Monitoring System (NARMS) displayed multi-drug resistance (24). NARMS found that 27% of *Salmonella* serotype Typhimurium isolates, and 8% of *Salmonella* serotype Heidelberg also displayed multi-drug resistance (24). MDR strains from other *Salmonella* serotypes of epidemiological importance, such as Agona, Dublin, Hadar, and Senftenberg have also been identified (24). In addition to the apparent increase of human salmonellosis infections by drug-resistant *Salmonella* with the resulting potential treatment difficulties, recent evidence also indicates that human infections with drug-resistant *Salmonella* are generally more severe than those caused by sensitive strains (53).

The human health impact of drug-resistant *Salmonella* is also exemplified by the fact that a number of salmonellosis outbreaks have been caused by MDR *Salmonella* (see Table 1.1 for examples) (6, 7, 92-94, 98, 109). These outbreaks have either been the result of the consumption of contaminated foods, or through direct contact with infected animals. In 2000, there was outbreak of *Salmonella* Typhimurium from contaminated milk in New Jersey. These isolates displayed resistance to 5 antibiotics: ampicillin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline (79). In 2002, there was an outbreak of multi-drug resistant Newport related to the consumption of contaminated beef products, affecting 47 people in 5 states (6). Isolates from this outbreak showed resistance to 9 antibiotics: amoxicillin/clavulanate, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. A few isolates also displayed kanamycin resistance and reduced sensitivity to ceftriaxone. More recently, there was series of outbreaks from December 2003 to late 2004, of *Salmonella* Typhimurium

TABLE 1.1. Drug-resistant *Salmonella* Outbreaks

Serotype	Resistance[a]	Implicated Vehicle	Location	Year	Reference
Blockley	CKST and KSTN	Unknown	Greece	1998	(94)
Heidelberg	KST	Stuffed ham	Maryland	1997	(91)
Newport	ACKSSuT	Ground Beef	California	1985	(91)
Newport	MDR-AmpC	Farm exposure	Massachusetts	1999	(91)
Newport	MDR-AmpC	Ground beef	Connecticut, New York, Michigan, Pennsylvania, Ohio	2002	(1)
Stanley	KTB	Alfalfa sprouts	Arizona	1995	(91)
Typhimurium	ACKSSuT	Raw milk	Arizona	1985	(87)
Typhimurium	ACKSSuT	Contact with infected animals	Idaho	1999-2000	(91)
Typhimurium	ACSSuT	Cheese	Washington	1997	(91)
Typhimurium	ACSSuT	Raw milk	Vermont	1997	(91)
Typhimurium	ACSSuT	Handling of rodents	Georgia, Illinois, Kentucky, Michigan, Minnesota, Missouri, New Jersey, North Carolina, Pennsylvania, and South Carolina	2003-2004	(2)
Typhimurium	AKSSuT	Milk	Illinois	1985	(91)
Typhimurium	AKSSuT	Farm exposure	Ohio	1998	(91)
Typhimurium	AKSSuT	Milk	Pennsylvania, New Jersey	2000	(91)
Typhimurium	TS	Salad Bar	Oregon	1984	(91)
Typhimurium DT104	ACSSuT + Quinolone (reduced susceptibility)	Pork	Denmark	1998	(68)
Typhimurium DT104	ACSSuT	Contact with infected animals	Minnesota and Washington	1999-2000	(100)
Typhimurium DT104	ACSSuT	Prepared lunch boxes	Japan	2003	(93)

[a]A, ampicillin; C, chloramphenicol; K, kanamycin; N, Naldixic Acid; S, streptomycin; Su, sulfamethoxazole; T, tetracycline; B, trimethoprim-sulfamethoxazole; MDR-AMPC, ACSSuT + amoxicillin/clavulanic acid, cephalothin, cefoxitin, ceftiofur, ceftriaxone (reduced susceptibility)

DT120 linked the handling of pet rodents (7). These isolates showed resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. Multi-drug resistant *Salmonella* thus clearly represent a growing health risk to the US population. New methods for the surveillance and control of multi-drug resistant *Salmonella* are necessary to prevent future outbreaks, or at least minimise their impact.

ANTIMICROBIAL RESISTANCE MECHANISMS IN *SALMONELLA*

The following portion of this review is separated into sections that cover the main classes of antimicrobials that are commonly used in the treatment of *Salmonella* infections in humans. Within each section we describe the mode of action of the antimicrobial, the mechanisms of resistance found in *Salmonella*, as well as the occurrence of resistance to a given antimicrobial class within members of the genus *Salmonella*. Table 1.2 contains a summary of each class of antimicrobial, genes commonly found to encode antimicrobial resistance, and *Salmonella* serotypes in which resistance genes have been commonly found.

Aminoglycosides. Aminoglycosides were first discovered in 1943, when streptomycin was isolated from *Streptomyces griseus*. Through the next decade other aminoglycosides, such as neomycin and kanamycin, were discovered and used to treat bacterial infections. Based on their chemical structure, aminoglycosides are divided into 4 classes, including: (i) streptidines, a class containing streptomycin and dihydrostreptomycin; (ii) streptamines, whose only clinically significant member is spectomycin; (iii) 4.5-distributed 2-deoxystreptamines, whose clinical efficacy is limited by their toxicity, and which are represented in clinical use only by neomycin; and (iv) 4,6-disubstituted 2-deoxystreptamines, that contain the most commonly used aminoglycosides including kanamycin, gentamicin, amikacin, and tobramycin (68).

TABLE 1.2. Summary of Antibiotic Resistance in *Salmonella*

Antibiotic	Mode of Action	Common Resistance Genes	Serotypes	References
Aminoglycosides	Inhibits protein synthesis	*aac(3)-IV, aac(3)-IVa, aacC2, strA, strB, aph(3')-IIA, aadA1, aadA2, aadB*	4,5,12:i:- , Agona, Anatum, Blockley, Bredeney, Derby, Give, Hadar, Heidelberg, Kentucky, London, Infantis, Saintpaul, Newport, Typhimurium	25, 34, 38, 48, 58, 61, 70, 77
Beta-lactams	Inhibitis cell wall formation	bla_{CMY-2}, bla_{CTX-M9} bla_{TEM-1}, bla_{TEM-53}, bla_{CARB2}, bla_{OXA-30}	Anatum, Agona, Blockley, Dublin, Enteritidis, Haadrt, Muenchen, Newprot, Stanley, Tyhimurium, Virchow	5, 8, 11, 25, 32, 42, 49, 88, 93, 95, 101
Chloramphenicol	Inhibits protein synthesis	*cat1, cat2, cmlA, floR*	Albany, Agona, Derby, Enteritidis, Haardy, Kiambo, Newport, Typhimurium	5, 16, 19, 25, 32, 33, 47, 66
Quinolones	Inhibits topoisomerase	*gyrA, gyrB, parC*[a]	Typhimurium, Enteritidis	12, 22, 50, 59
Tetracyclines	Inhibits protein synthesis	*tet(A), tet(B)*	Agona, Anatum, Blockley, Bredeney, Colorado, Derby, Dublin, Enteritidis, Give, Haardt, Hadar, Heidelberg, Infantis, Orion, Seftenberg, Typhimurium	25, 39, 47, 77
Sulfonamides	Inhibits dihyrdropteroate synthetase	*sul1, sul2, sul3*	4,5,12:i:- Agona, Albany, Anatum, Bradenburg, Derby, Djugu, Enteritidis, Hadar, Heidelberg, Orion, Rissen, Typhimurium	9, 25, 33, 34, 45
Trimethoprim	Inhibits dihydrofolate reductase	*dhfr1,dhfr12, dfrA1, dfrA5, dfrA14, dfrA15, dfrA16*	4,5,12:i:-, Agona, Albany, Derby, Djugu, Hadar, Neport, Rissen, Typhimirium	9, 25, 31, 33, 34, 38, 48, 57, 62

[a]Resistance is mediated by point mutations in these genes

Aminoglycosides are effective in treating infections caused by gram-negative bacilli and are usually used in combination with other antibiotics to ensure a broad-spectrum of action (46). They bind to conserved sequences within the 16S rRNA of the 30S ribosomal subunit (68). This binding leads to codon misreading and translation inhibition. Most aminoglycosides are bactericidal, with the exception of spectinomycin whose mode of action is bacteriostatic (68).

Primary mechanisms for bacterial resistance to aminoglycosides include: (i) decreased uptake of the antibiotic; (ii) modification of the antibiotic; and (iii) modification of the ribosomal target of the antibiotic. While efflux pumps, which remove an antibiotic from within the cell, have been found to play a role in aminoglycoside resistance in other enterobacteria, such as *E. coli* (2, 86), and play role in the resistance of *Salmonella* to other antibiotics, this mechanisms has yet to be associated with aminoglycoside resistance in *Salmonella*. Ribosomal modification has not been reported as a mechanism in *Salmonella* aminoglycoside resistance. Aminoglycoside resistance in *Salmonella* is generally associated with the expression of a plasmid-mediated aminoglycoside-modifying enzymes (43, 49). These enzymes fall into three groups that are named according to the types of reactions they catalyze including: acetyltransferases, phosphotransferases, and nucleotidyltransferases. A comprehensive review of aminoglycosides is provided by Shaw et al. (90).

Aminoglycoside acetyltransferases are enzymes that primarily modify aminoglycoside amino groups (68, 90). These enzymes are subdivided into four groups based on the areas that they modify: AAC(1), AAC(2'), AAC(3), and AAC(6') (68). Genes encoding these enzymes are typically designated *aac* (97). Many variants have been found in a wide range of *Salmonella* subtypes; including Newport (35), Agona (75), Typhimurium (26), Typhimurium var. Copenhagen (40), Kentucky (61), and 4,5,12:i:- (50). *aac* genes have been found as part of *Salmonella* genomic islands

(35), integrons (50, 61, 81), and plasmids (50). Aminoglycoside acetyltransferases provide resistance to gentamicin, tobramycin, and kanamycin (68).

Aminoglycoside phosphotransferases are enzymes that catalyze the ATP-dependent phosphorylation of specific aminoglycoside hydroxyl groups (68, 90). This group is divided into subgroups whose classification depends on the specific site of phosphorylation. Subgroups APH(3") and APH(6), both of which provide resistance to streptomycin (68), have been found encoded on plasmids harbored by *Salmonella* (43). Most genes encoding these enzymes are designated as *aph* (97), though it should be noted that the genes *aph(3")-Ib* and *aph(6)-Id* are commonly referred in the literature as *strA* and *strB*, respectively (65, 90). Genes from both families have been found in *Salmonella* serotypes Agona, Anatum, Blockely, Bredeney, Derby, Give, Hadar, Heidelberg, London, Saintpaul, and Typhimurium (65, 82). Genes encoding enzymes of the APH(3') subgroup, provide resistance to kanamycin and neomycin (68), and have been found in several *Salmonella* subtypes such as Derby (26), Haardt (26), Enteritidis (26), Typhimurium (43), and Typhimurium var. Copenhagen (40).

The final group of enzymes providing aminoglycoside resistance are nucleotidyltransferases (68, 90). These enzymes also target the hydroxyl groups and are divided into several subgroups based on the site of modification. Genes encoding nucleotidyltransferases are usually designated *aad* (97), though some are also designated as *ant*. The gene *aadA*, also referred to as *ant(3")* (90), provides streptomycin resistance in *Salmonella* (68). There are several variants of this gene and they have been found in serotypes Agona, Anatum, Bredeney, Derby, Enteritidis, Give, Heidelberg, Saint Paul, and Typhimurium (26, 65, 82). The gene *aadB*, also known as *ant(2')-Ia* (90), confers resistance gentamicin and tobramycin (68). It has been found in serotypes Typhimurium and Typhimurium var Copenhagen (22, 39,

40). Both *aadA* and *aadB* have been found in integron-borne gene cassettes (22, 82, 107).

Beta-Lactams. There are three major groups of beta-lactams: penicillins, cephalosporins, and carbapenems. They all work by interfering with a group of seven proteins, known as penicillin-binding proteins (PBPs). These proteins are involved in the synthesis of peptidoglycan, an essential component of the bacterial cell wall. Beta-lactams are generally considered bactericidal, however the activity varies among beta-lactams, organisms, and target PBP. In *Salmonella* and *E. coli*, it appears that inhibition of the essential PBPs, 1 through 3, leads to bactericidals activity (80, 88). Due to their wide clinical use, resistance to penicillins, like ampicillin and methicillin, has become common (5), and in response a second class of beta-lactams, called cephalosporins, were developed. Cephalosporins are similar in structure to penicillins, but have a 6, rather than 5, member beta-lactam ring, and an extra functional group (54). These changes provide cephalosporins with a broader range of activity and greater stability in the presence of beta-lactamases. There are four generations of cephalosporins, and each progressive generation is effective against a broader range of organisms (54). Cephalosporins have become a popular form of treatment, but with increased use has come increased resistance. The latest group of beta-lactams to be discovered is the carbapenems. They are a cross between the 5-member beta-lactam ring of penicillins and the extra functional group of cephalosporins, and are sometimes paired with beta-lactamase inhibitors (68). Carbapenems have a much larger range of activity against both gram-negative and gram-positive bacteria than other beta-lactams, and are also more stable against beta-lactamases. For this reason, their use is generally reserved for severe cases that involve multi-drug resistant bacteria, however there are already cases of *Salmonella* isolates that posses resistance to carbapenems

such as imipenem (11, 72). A detailed account of the structure and function of beta-lactams is provide by Mascaretti (68).

For beta-lactams to reach their PBP targets, they must first traverse the bacterial cell wall. This passage is facilitated by two porins, OmpC and OmpF (55). While porin loss/modification is an uncommon mechanism for beta-lactam resistance in *Salmonella*, cases have been documented where decreases in either OmpF (16) or OmpC (70) porin levels have lead to observable increases in resistance. One study on *Salmonella envB* mutants, which have reduced porin content, found that the reduction in OmpF and OmpD porin expression actually lead to decreased resistance to most beta-lactams other than mecillinam and imipenem, though this may have been due to other effects of the *envB* mutation on the isolates (80).

The most common mechanism of resistance to beta-lactams in *Salmonella*, is the secretion of a beta-lactamase. These enzymes work by hydrolyzing the ß-lactam ring structure, yielding beta-amino acids with no antimicrobial activity. The genes encoding for beta-lactamases produced by *Salmonella* are typically carried on plasmids (68), though most of these genes are chromosomally encoded in other bacterial species. There are several different classification schemes for the characterization of beta-lactamases, but for the purposes of this review, Ambler's classification scheme, which is based on primary structure and amino acid sequence identity, will be used (68).

In general, class A beta-lactamases are the most commonly found class of beta-lactamases in *Salmonella*. They are plasmid encoded, and provide a range of resistance against penicillins, early generation cephalosproins, and carbapenems. There are several different gene families encoding for enzymes in this class, with TEM being the most prevalent among *Salmonella*. The gene $bla_{\text{TEM-1}}$ has been found in subtypes Enteritidis, Dublin, Haadrt, Muenchen and Tyhimurium (26, 44). A

variant, *bla*$_{TEM-52}$, has been found in serotype Enteritidis, Blockley, Panama, and Typhimurium (95, 102, 110). Other class A beta-lactamase genes such *bla*$_{PSE-1}$, which is also known in the literature as *bla*$_{CARB-2}$ (64), have also been found in a number *Salmonella* subtypes (64, 103). Recently, a class A beta-lactamase gene, *bla*$_{KPC-2}$, which provides resistance to imipenem, was found in a *Salmonella* serotype Cubana isolate (72). The emergence of cefotaximases (CTX-M), which are class A beta-lactamases conferring resistance to ampicillin and cephalosporins, is an important trend to watch (12). *Bla*$_{CTX-M}$ variants have been identified in *Salmonella* serotypes Anatum, Enteritidis, Stanley, Typhimurium, and Virchow isolates from Europe (12, 104).

The second most common class of beta-lactamases is class C beta-lactamases. These are typically encoded by chromosomal *ampC* genes and provide resistance against cephalosporins such as cefoxitin and ceftiofur. *Salmonella* carries no chromosomal *ampC* gene, instead these genes are harbored in plasmids carried by *Salmonella* (74, 106). Current research is primarily focused on the presence of *bla*$_{CMY-2}$, which has been associated with resistance to ceftiofur (3, 106). Ceftiofur is a 3rd generation cephalosporin closely related to ceftriaxone. The spread of this gene is a public health concern as ceftriaxone is the drug of choice for the treatment of *Salmonella* infections in children. Several *Salmonella* serotypes such as Typhimurium, Agona, and Newport (3, 33), have been found to carry this gene. Other class C genes and CMY variants, such as *bla*$_{CMY-4}$ (11) and *bla*$_{CMY-7}$ (51), have been found, but are not as common in *Salmonella*.

Class B corresponds to metallo-beta-lactamases. These enzyme provide resistance to all beta-lactam antibiotics, including carbapenems such as imipenem (68). Metallo-beta-lactamases are usually chromosomally encoded, though plasmid-

mediated class B beta-lactamases, such as IMP-1 and VIM-1, do exist (68). Class B beta-lactamases are not commonly found in *Salmonella*.

Class D beta-lactamases are uncommon among *Salmonella*. This class of enzymes provides resistance to lactams closely related to oxacillin, such as cloxacillin and methicillin. The gene $bla_{\text{OXA-1}}$ was found in a *Salmonella* serotype Paratyphi (20) and $bla_{\text{OXA-30}}$ has been found in serotypes Muenchen and Typhimurium (9, 44, 51).

Chloramphenicols. Chloramphenicols were once the drug of choice for the treatment of typhoid fever (68). Production of chloramphenicols by *Streptomyces venezuelae* was discovered in 1947. Chloramphenicol works by binding to the peptidyltransferase center of the 50s ribosomal unit, thus preventing formation of peptide bonds (68). Chloramphenicol's broad range activity against gram-positive and gram-negative bacteria and its ability to cross the blood-brain barrier make it a powerful choice for the treatment of systemic infections. Its toxicity, which can lead to bone marrow damage and aplastic anemia (68, 111), and widespread resistance, have limited chloramphenicol use to occasions where the risk of the infection, like bacterial meningitis, is greater than the risk of side effects from the drug (68).

Chloramphenicol resistance in *Salmonella* is conferred through two mechanisms: (i) the enzymatic inactivation of the antibiotic via chloramphenicol O-acetyltransferase (CAT); and (ii) the removal of the antibiotic via an efflux pump. The genes encoding for CAT are plasmid-borne (49) and commonly found in *Salmonella* Typhi isolates (68, 89). CAT genes, such as *cat1* and *cat2*, have also been found in non-typhoidal *Salmonella* serotypes like Derby, Enteritidis, Haardy, and Typhimurium (26, 49).

Chloramphenicol efflux pumps in *Salmonella* have been reported to be encoded by two closely related genes, *cmlA* (20) and *floR* (105). *floR* appears to be very widespread in *Salmonella*, where as *cmlA* is less widely distributed. Serotypes

Albany, Agona, Kiambo, Newport, Typhimurium, Typhimurium var Copenhagen have been found to carry *floR* (3, 17, 20, 33, 34, 71). It is a highly mobile gene, as it has been found *Salmonella* genomic islands (17, 34, 103), as well as in many different plasmids (29, 33, 71), and appears associated with multi-drug resistance (3, 33) most likely due to its presence on plasmids carrying multiple resistance genes.

Quinolones. Quinolones are synthetic, bactericidal antimicrobials. Nalidixic acid became the first quinolone approved for medical use in 1962 (68). Several generations of quinolones have been developed, with each new generation showing improved action against bacterial infection. The early-generation quinolones target DNA gyrase, while the late-generation quinolones target DNA topoisomerase IV (68, 108). Quinolone's mode of action is quite complex, and not completely understood (68). Research indicates that while quinolones target topoisomerases, the antibiotic does not actually bind to the topoisomerase, but to the double stranded DNA in the topisomerase complex (91). For further information on quinolones and their mode of action, the reader is referred to more comprehensive reviews (68, 108).

While there are documented cases of *Salmonella* with resistance to nalidixic acid and low-level resistance to other quinolones (18, 73), high-level resistance to quinolones is still rare (23, 78). Quinolone resistance in *Salmonella* has been tied to two mechanisms. The first is target mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB*, the two genes which encode for the subunits of DNA gyrase, and in the *parC* subunit of topisomerase IV (13, 23, 30, 52, 62). The second mechanism involves changes in the expression of the AcrAB-TolC efflux system, mostly due to mutations in the regulator genes of this system, that result in over expression of this efflux system (4, 13, 14, 30, 57, 62, 77), which results in decreased quinolone sensitivity. No one mutation confers high-level resistance to quinolones, rather it is an accumulation of all these mutations that provides resistance

(52). The fact that *Salmonella* must acquire multiple, unlinked mutations, and that some of those mutations reduce fitness, particularly those involved in the regulation of the efflux pump (45), may explain why this kind of resistance is so infrequent.

In some bacterial species, such as *E. coli* (101) and *Klebsiella pneumoniae* (100), quinolone resistance has also been linked to the expression of a plasmid-mediated gene called *qnr* (63). The gene expresses a protein that appears to bind to DNA-gyrase and protect it from quinolone inhibition (63). Research conducted on plasmids harboring *qnr* showed that it could be transferred from other bacterial species to *Salmonella* via conjugation (67). While documented cases of plasmid-mediated quinolone resistance in *Salmonella* are rare, a recent study indicates the spread of such plasmids to *Salmonella* has occurred (27). The appearance of plasmid-mediated quinolone resistance in *Salmonella* is a very important emerging public health concern. Plasmids harboring *qnr* have also been found to harbor other resistance genes (63), suggesting that the treatment of infections by *Salmonella* strains with this plasmid may be increasingly difficult.

Tetracyclines. Tetracyclines were discovered in the 1940's. The first tetracycline, chlorotetracycline, was isolated from *Streptomyces aureofaciens* (68). Tetracyclines were popular due their minimal side effects and broad-spectrum of activity. They were effective against most bacteria, chlamydias, mycoplasms, and even some protozoa (28, 68). Tetracyclines act by preventing the binding of tRNA to the A site of the 30S ribosomal subunit, thus inhibiting protein synthesis (68). Unfortunately, like many popular forms of antibiotics, the rise of resistant bacteria has severely limited the use of these drugs. For a more in depth review of tetracycline structure and function, the reader is directed to the review by Chopra and Roberts (28).

Tetracycline resistance in *Salmonella* is attributed to production of an energy-dependent efflux pump to remove the antibiotic from within the cell. Other

mechanism of resistance, such as modification of the ribosomal target and enzymatic inactivation of tetracycline, have been documented in other bacterial species but have yet to be reported in *Salmonella* (28, 68).

There are at least thirty-two different genes that confer resistance to tetracycline and oxytetracycline (28, 68). Of these, *tet(A), tet(B), tet(C), tet(D), tet(G),* and *tet(H)* have been found in *Salmonella* (21, 28, 40). The most common of these is *tet(A).* It has been found in *Salmonella* genomic island 1 (21), harbored on integrons (19), and present on transferable plasmids (42, 44, 82). It has been found in serotypes Agona, Anatum, Blockley, Bredeney, Colorado, Derby, Enteritidis, Give, Haardt, Hadar, Heidelberg, Infantis, Orion, Seftenberg, and Typhimurium (26, 82). The gene *tet(B)* is also fairly common, it has been found in serotypes Agona, Dublin, Choleraesuis, Heidelberg, Typhimurium (26, 41, 49). Like *tet(A), tet(B)* has also been located on transferable plasmids (49). These genes appear to be easily transferred and wide spread among *Salmonella*. They also tend to be found in isolates that display multi-drug resistance (21, 26, 82), making them an important marker in identifying potentially serious *Salmonella* infections.

Sulfonamides and Trimethoprim. Though initially prescribed separately, sulfonamides and trimethoprim have been used in combination for the treatment of bacterial infection since the late 1960's (68). They are bacteriostatic antibiotics that act by competitively inhibiting enzymes involved the synthesis of tetrahydrofolic acid. Sulfonamides inhibit dihyrdropteroate synthetase (DHPS), while trimethoprim inhibits dihydrofolate reductase (DHFR) (68). The combination of sulfonamide and trimethoprim has been a popular form of treatment for decades, and while still effective in the treatment of bacterial infections, resistance among *Salmonella* has emerged.

Sulfonamide resistance in *Salmonella* has been attributed to the presence of an extra *sul* gene which expresses an insensitive form of DHPS (10, 68). Three main genes have been indentified; *sul1, sul2,* and *sul3*. The gene *sul1* has been found in a wide range of serotypes such as Agona, Albany, Derby, Djugu, Enteritidis, Hadar, Heidelberg, Orion, Rissen, and Typhimurium (10, 26, 34, 35). This gene is often associated with class I integrons that contain other resistance genes (48, 59, 87). These integron-borne gene cassettes have been found on transferable plasmids (49) and as part of *Salmonella* genomic island variants (17, 34, 35). While sometimes found in isolates also harboring *sul1* (10, 26), *sul2* appears to associated with plasmids, but not class I integrons (10). Serotypes Agona, Enteritidis and Typhimurium isolates have been reported to carry *sul2* (26). *sul3* has only been recently identified in *Salmonella*, and it has been associated with plasmids (47) and class I integrons in *Salmonella* (10), suggesting there may be further dissemination of this gene within *Salmonella* populations. *sul3* has already been found in serotypes 4,5,12:i:-, Agona, Anatum, Bradenburg, Heidelberg, Rissen, and Typhimurium (10, 47).

Like sulfonamide resistance, trimethoprim resistance is attributed to the expression of DHFR that does not bind trimethoprim (68). There are many variants of the *dhfr*/*dfr* genes that encode this resistance (1, 68), such as *dhfr1*, *dfrA1*, *dhfr12* (26, 34, 60). Serotypes known to carry trimethoprim resistance genes are 4,5,12:i:-, Agona, Albany, Derby, Djugu, Hadar, Neport, Rissen, and Typhimirium (10, 32, 34, 35, 50, 66). These genes have been found as part of integron-borne gene cassettes also associated with *sul1* and *sul3* (10), on transferable plasmids carrying other resistance genes (50, 99), and *Salmonella* genomic islands (34, 35).

TRANSMISSION OF ANTIBIOTIC RESISTANCE

There are two mechanisms that are primarily responsible for the spread of antibiotic resistance within *Salmonella* populations. The first is clonal spread of antibiotic resistant *Salmonella* subtypes, especially in environments where their resistance provides them with a selective advantage. The most well known multi-drug resistant clonal group is *Salmonella* serotype Typhimurium DT104. It has been found world-wide (37, 83, 85, 96), and has been responsible for numerous outbreaks (58, 73). MDR Newport is another drug-resistant clonal group that has recently appeared in the United States (24, 38, 84). It has been suggested that antibiotic use on farms may be selecting for and promoting the spread of these multi-drug resistant clonal groups (6). Effective monitoring and prudent use of antibiotics is necessary to curb the spread of these *Salmonella* strains and to prevent the emergence of new antibiotic resistant clonal groups.

The second form of antibiotic resistance transmission is horizontal gene transfer of resistance genes between *Salmonella* subtypes. In *Salmonella*, plasmids and class I integrons are primarily responsible for such transfers. Genes conferring resistance to aminoglycosides (75, 90), beta-lactams (15, 33, 56), choramphenicols (29, 33), tetracyclines (50, 82), sulfonamides (10, 47), and trimethoprim (1, 99) have all been found on several different plasmid types. Many of these plasmids carry multiply antibiotic resistance genes that are transferable to other *Salmonella* subtypes and other bacterial species (33, 49, 50, 99).

Class I integrons are an important mechanism for antibiotic resistance transmission. They are known to be composed of: (i) a conserved 5' region consisting of the integrase gene, *int*, and a promoter; (ii) a conserved 3' region consisting of ethidium bromide/quaternary ammonium compound resistance locus, *qacE*? *1*, and the sulfonamide resistance gene *sul1*; and (iii) a gene cassette containing multiple

antibiotic resistance genes in varying combinations (59, 87). Integrons have been found as part of plasmids (49, 50) and transposons (82, 99) carried by *Salmonella*. Class I integrons are primary components of *Salmonella* genomic islands, which are the genetic elements responsible for multi-drug resistance in some isolates of *Salmonella* serotypes Agona, Albany, Newport, and Typhimurium DT104 (17, 32, 34, 35, 60). Recent research has shown that these genomic islands are in fact complex class I integrons (31). They can be horizontally transferred when in the presence of a conjugative helper plasmid, and have been found in multiple *Salmonella* subtypes (31), indicating that further dissemination of this multi-drug resistant element among *Salmonella* is likely.

CONCLUSIONS

Antimicrobial resistance genes are typically found on highly mobile genetic elements, such as integrons and plasmids, which are readily transferred between *Salmonella* strains and other bacterial species. These multi-drug resistant *Salmonella* strains have been found worldwide, and represent a growing public health concern. A key component in controlling the spread of multi-drug resistance *Salmonella* is the development of a fast, accurate, and consistent monitoring system. Current typing methods used for monitoring *Salmonella* are serotyping, pulse field gel electrophoresis (PFGE), and multi-locus sequence typing (MLST). Of the three, PFGE provides the most discrimination among isolates, but is also the hardest to compare between laboratories. MLST is the most consistent across laboratories, and provides more subtype differentiation than traditional serotyping, but it has yet to see widespread use. While any of these methods may help us identify known multi-drug resistant *Salmonella* subtypes, by themselves, they do not tell us when a previously sensitive *Salmonella* subtype acquires drug resistance. In the future it will be important to

incorporate technologies, like DNA microarrays, to quickly screen clinical isolates for the presence of antimicrobial resistance genes and elements. This new, combined monitoring system, as well as the implementation of antimicrobial treatment programs that discourage the maintenance of resistance genes in the *Salmonella* population, will be necessary to curb the emergence and spread of multi-drug resistance *Salmonella*.

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CHAPTER 2

CEFTIOFUR RESISTANT *SALMONELLA* ISOLATED FROM DAIRY FARMS REPRESENT MULTIPLE WIDELY DISTRIBUTED SUBTYPES THAT EVOLVED BYIDEPENDENT HORIZONTAL GENE TRANSFER*

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ABSTRACT

Salmonella is the leading cause of known foodborne bacterial infections in the US, 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 $bla_{\text{CMY-2}}$, a beta-lactamase gene associated with resistance to cephalosporins. Our data indicate that (i) resistance to ceftriaxone and ceftiofur were highly correlated with the presence of $bla_{\text{CMY-2}}$; (ii) ceftiofur resistant *Salmonella* were geographically widespread as shown by their isolation from farms located throughout New York state; (iii) ceftiofur resistant *Salmonella* 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 $bla_{\text{CMY-2}}$ allele and by clonal spread of ceftiofur resistant subtypes.

INTRODUCTION

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 bacterial foodborne illness, and the leading cause of bacterial foodborne hospitalizations and deaths (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* has lead to a decrease in the efficacy of these treatments (2). Currently, fluoroquinolones and third-generation 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* producing ß-lactamases conferring resistance to third-generation cephalosporins have been isolated from clinical patients (11, 19, 38).

Of particular concern is the appearance of *Salmonella* with decreased susceptibility to ceftiofur (1, 5, 15, 38, 41). Ceftiofur is a third-generation cephalosporin with broad range activity against both gram-positive and gram-negative bacteria. It is the only third-generation cephalosporin approved in the US 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 account for 25% of all *Salmonella* infections in the US (6). Beef and dairy products accounted for 10% of reported foodborne *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 transmission and evolution of ceftiofur and ceftriaxone resistant *Salmonella* are needed.

The most common mechanism of cephalosporin resistance is the production of ß-lactamases. Cephalosporins are semisynthetic antibiotics originally derived from cephalosporin C, a naturally occurring antimicrobial produced by *Cephalosporium acremonium*. Like other ß-lactams, such as penicillin and ampicillin, cephalosporins act by targeting various penicillin-binding proteins (PBPs) 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 ß-lactam ring. ß-lactamases confer resistance by hydrolyszing the ß-lactam ring, producing ß-amino acids with no antimicrobial activity (20). Third-generation cephalosporins, like ceftiofur and ceftriaxone, are prescribed to treat *Salmonella* infections due to their increased activity against Gram-negative bacteria and the presence of oxyimmino side chains that provide increased ring stability in the presence of ß-lactamases (18, 20).

Despite the effectiveness of third-generation cephalosporins in combating *Salmonella* infections, resistant subtypes have emerged. Unlike other enterobacteria, *Salmonella* possess no chromosomal ß-lactamase gene (22). Instead, resistance to ceftiofur and ceftriaxone in *Salmonella* has been traced to a plasmid-encoded AmpC-like ß-lactamase, CMY-2 (7, 38, 39). AmpC ß-lactamases belong to class C of Ambler's structural characterization, meaning they are active-site serine ß-lactamases, and are typically encoded by chromosomal *bla* genes (20). Plasmid-borne *ampC* genes appear to be derived from chromosomal genes; for example $bla_{\text{CMY-2}}$ 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 (RFLP) analysis and southern blotting have shown that $bla_{\text{CMY-2}}$ 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 current 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 as well as one 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-APHIS-VS, Ames, IA).

Antibiotic resistance profiles. To characterize antimicrobial resistance of the isolates, Standard National Antimicrobial Resistance Monitoring System (NARMS) (34) panels were performed at the NY State Animal Health Diagnostic Center (AHDC, 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, Ontario Candada). The antimicrobial agents tested included Amikacin (AMK), Amoxicillin/Clavulanic Acid (AMC), Ampicillin (AMP), Cefoxitin (FOX), Ceftiofur (CEF), Ceftriaxone (CRO), Chloramphenicol (CHL), Ciprofloxacin (CIP), Gentamicin (GEN), Kanamycin (KAN), Nalidixic Acid (NAL), Streptomycin (STR), Sulfizoxazole (SUF), Tetracycline (TET), and Trimethoprim/Sulphamethoxazole (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 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 MLST are presented in Table 2.1. 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 2.1). 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 $bla_{\text{CMY-2}}$ and *floR*, using the conditions and primers listed in Table 2.1. For $bla_{\text{CMY-2}}$ positive isolates, a full-length PCR amplicon was created using *ampC* primers (Table 2.1).

TABLE 2.1. PCR primers and conditions

Gene	Primers	PCR conditions[a]	Reference
manB	F: 5'-CAT AAY CCG ATG GAC TAC AAC G-3' R: 5'-ACC AGC AGC CAC GGG ATC AT-3'	95°C/9:30 min (1x); 95°C/45 s, TD 55–45°C/45 s, 72°C/60 s (40x); 72°C/7 min (1x)	32
mdh	F: 5'-GAT GAA AGTCGC AGT CCT CG-3' R: 5'-TAT CCA GCA TAG CGT CCA GC-3'	95°C/9:30 min (1x); 95°C/45 s, TD 58-48°C/45 s, 72°C/60 s (40x), 72°C/7 min (1x)	32
fimA	F: 5'- TCA GGG GAG AAA CAG AAA ACT AAT -3' R: 5'- TCC CCG ATA GCC TCT TCC 3-3'	95°C/9:30 min (1x); 95°C/45 s, 57°C/45 s, 72°C/60 s (35x); 72°C for 7 min (1x)	32
bla_{CMY-2}	F: 5'-TGG CCA GAA CTG ACA GGC AAA-3' R: 5'-TTT CTC CTG AAC GTG GCT GGC-3'	95°C/9:30 min (1x); 95°C/45 s, 60°C/45 s, 72°C/60 s (40x); 72°C/7 min (1x)	26
ampC	F: 5'-AAC ACA CTG ATT GCG TCT GAC-3' R: 5'-CTG GGC CTC ATC GTC AGT TA-3'	95°C/9:30 min (1x); 95°C/45 s, 60°C/45 s, 72°C/60 s (40x); 72°C/7 min (1x)	26
floR	F: 5'-CTG AGG GTG TCG TCA TCT AC -3' R: 5'-GCT CCG ACA ATG CTG ACT AT-3'	95°C/9:30 min (1x); 95°C/30 s, 55°C/60 s, 72°C/60 s (40x); 72°C/7 min (1x)	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).

This *ampC* amplicon was purified as described above and sequenced using both ampC and bla_{CMY-2} forward and reverse primers (Table 2.1).

MLST typing. The MLST typing 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 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 one nucleotide. Allele assignments were performed to be consistent with allelic types previously reported by Sukhnanand et al. (33), i.e., 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 (ML) 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 *E. 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 linked to the reference for this manuscript and 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, a MLST typing scheme, as well as phenotypic and PCR-based methods to detect the presence of selected antibiotic resistance genes, were 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 $bla_{\text{CMY-2}}$; (ii) ceftiofur resistant *Salmonella* are geographically widespread as shown by their isolation from farms located throughout New York state; (iii) ceftiofur resistant *Salmonella* 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 $bla_{\text{CMY-2}}$ allele and by clonal spread of ceftiofur resistant subtypes.

Resistance to ceftriaxone and ceftiofur is highly correlated with the presence of $bla_{\text{CMY-2}}$. 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 $bla_{\text{CMY-2}}$, and 17 of these isolates also showed at least intermediate resistance to ceftriaxone, as defined by Sensititre® system analysis. There were no ceftiofur sensitive isolates that harbored $bla_{\text{CMY-2}}$. In addition, 24 isolates harbored *floR*, which encodes for 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 $bla_{\text{CMY-2}}$ (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 four did not carry plasmids that were typable using this method. Isolates carrying $bla_{\text{CMY-2}}$ showed a range of MIC values for ceftriaxone (Supplemental Table S1). Previous studies on *ampC*-mediated antibiotic resistance in other enterobacteriaceae did not show 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 $bla_{\text{CMY-2}}$ was also associated with multiple drug resistance. All 19 isolates harboring $bla_{\text{CMY-2}}$, showed resistance to seven other antibiotics, including ampicillin, amoxicillin, cefoxitin, chloramphenicol, sulfizoxazole, streptomycin, and tetracycline. In addition, 18 of these isolates also showed resistance to kanamycin, and all five ceftiofur resistant *Salmonella* Agona showed resistance to trimethoprim/sulphamethoxazole. Similar antibiotic resistance patterns have been noted in other studies (5, 7). Specifically, Carattoli et al. (5, 7) found a resistance profile similar to that found in our MLST type 2 *Salmonella* Agona in a human *Salmonella* Typhimurium isolate from Oregon and Chen et al. (5, 7) also reported similar resistance profile in *Salmonella* Agona isolates obtained from ground turkey in the US. Carattoli et al. (5, 7) also found a human *Salmonella* Typhimurium isolate from New York state and a human *Salmonella* Newport isolate from Kansas with resistance profiles similar to a MLST type 6 *Salmonella* Typhimurium reported here.

Multiple drug resistance was not as common in isolates lacking $bla_{\text{CMY-2}}$. Half of the isolates lacking $bla_{\text{CMY-2}}$ were sensitive to all antimicrobials tested, and three showed resistance to three or fewer antimicrobials tested. The remaining seven

isolates showed resistance to ampicillin, kanamycin, sulfizoxazole, streptomycin and tetracycline. Of these isolates, two also showed resistance to chloramphenicol and intermediate resistance to amoxicillin, and one showed resistance to chloramphenicol and gentamycin.

Ceftiofur resistant *Salmonella* are geographically widespread. Ceftiofur resistant *Salmonella* were isolated from farms across New York State (Fig. 2.1) (36). *Salmonella* harboring $bla_{\text{CMY-2}}$ 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 D.C. metropolitan area (7). In addition, a human outbreak of *Salmonella* Newport, which were resistant to ceftiofur, was reported in 2002 in 5 states including New York, Michigan, Pennsylvania, Ohio, and Connecticut (41). Ceftiofur resistant *Salmonella* thus appear to be widespread within the US, and may pose a growing problem for effective antibiotic treatment of *Salmonella* infections (15).

Ceftiofur resistant *Salmonella* represent multiple distinct subtypes and evolutionary lineages. MLST grouped the 39 isolates tested into six distinct MLST types, encompassing five different serotypes (Table 2.2). Serotypes Schwarzengrund and Anatum each represented a single MLST type, whereas Agona could be differentiated into two MLST types. MLST types 8 and 6 contained both Typhimurium and Typhimurium var. Copenhagen serotypes. The difficulty in differentiating these two serotypes with a MLST scheme was expected due to their high genetic similarity (28). Of the six MLST types, only MLST type 2 serotypes Agona, MLST type 6 serotype Typhimurium and MLST type 8 serotype Typhimurium var. Copenhagen contained isolates with ceftiofur resistance. While these serotypes have previously been found among ceftiofur resistant *Salmonella* isolated from cattle,

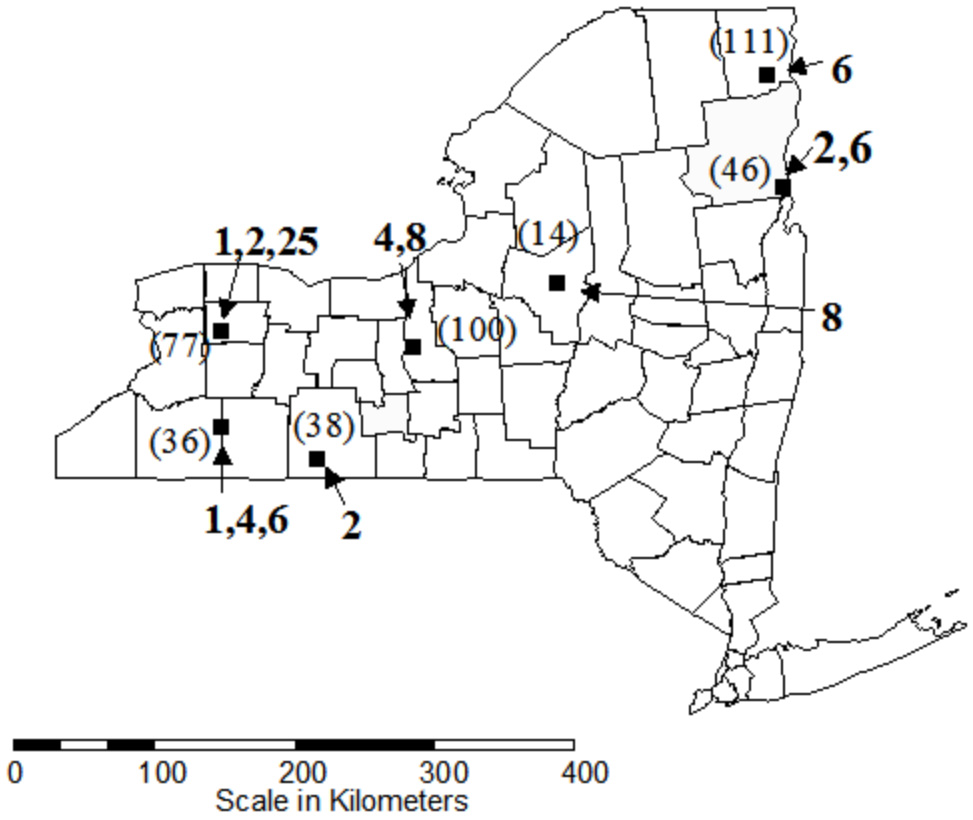

FIGURE 2.1. Distribution of MLST types across New York dairy farms. Numbers in parenthesis indicate farm number and bold numbers indicate MLST types isolated on the respective farms.

TABLE 2.2. 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 var. Copenhagen (3)	4	5	5	6
Typhimurium (2)	4	5	7	8
Typhimurium var. 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. (33)

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 *bla*$_{CMY-2}$ (Fig. 2.2). Evolutionary analysis of the 39 isolates revealed that they formed three strongly supported clades including one containing *Salmonella* Typhimurium and Typhimurium var. Copenhagen isolates (MLST types 6 and 8), one containing *Salmonella* Agona isolates (MLST types 1 and 2), and one containing *Salmonella* Schwarzengrund isolates (MLST type 4). The sole Anatum isolate grouped close to the Schwarzengrund clade, but its branch was not supported by a high bootstrap value (<50). Within the *Salmonella* 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 Typhimurium/Typhimurium var. Copenhagen clade contained isolates that carried *bla*$_{CMY-2}$, neither the Schwarzengrund nor the 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 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

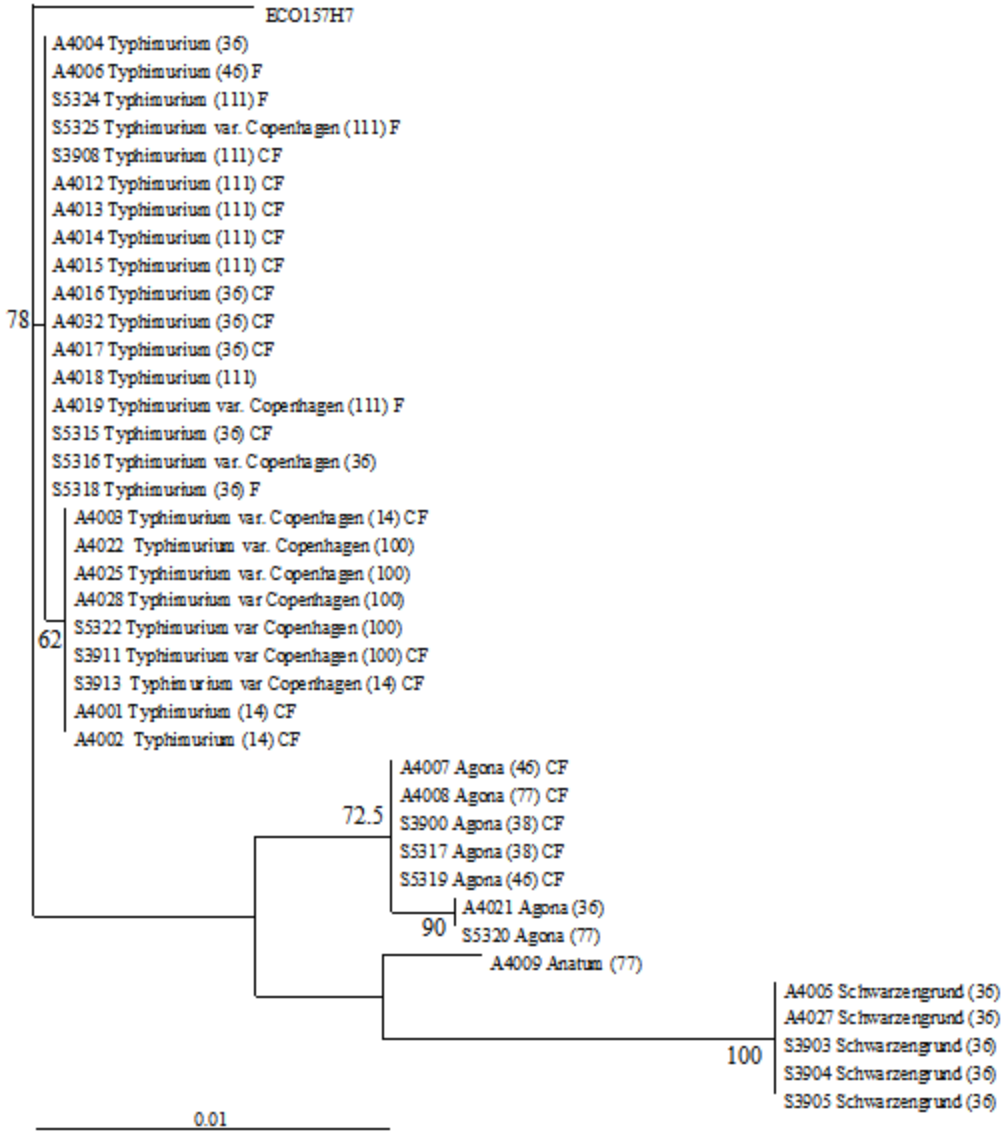

FIGURE 2.2. Phylogenetic tree of *Salmonella* isolates based on the concatenated *manB*, *mdh*, and *fimA* sequences. The phylogenetic tree was built using the maximum likelihood method and the TrN+G model, which was selected by MODELTEST as the best model. The outgroup branch length was collapsed for easier viewing. Bootstrap values > 50.0 are indicated at the node of the branch. Numbers in parenthesis indicate farm number, C indicates presence of bla_{CMY-2}, and F indicates presence of *floR*. The scale bar indicates relative sequence distance.

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* 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* isolated on these farms. The high level of genotypic and 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* Agona with a very similar antibiotic resistance profile was isolated from turkey meat in the D.C. area (7), suggesting that this clonal group may be present in other US regions. The fact that this Agona subtype is easily identifiable via an MLST typing 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 isolates from farm 100 was classified into this clonal group (Table 2.3). Other 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

TABLE 2.3. Serotype, MLST, and antibiotic resistance profiles of *Salmonella* isolates

FSL ID	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 var. 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 var. Copenhagen	111	6	ChlSufStr
FSL A4-019	Typhimurium var. 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 var. Copenhagen	100	8	AmpKanSufStrTet
FSL A4-025	Typhimurium var. Copenhagen	100	8	AmpKanSufStrTet
FSL A4-028	Typhimurium var. Copenhagen	100	8	AmpKanSufStrTet
FSL S5-322	Typhimurium var. 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 var. Copenhagen	14	8	AmpAmcFoxCefCro*ChlKanSufStrTet
FSL S3-913	Typhimurium var. Copenhagen	14	8	AmpAmcFoxCefCro*ChlKanSufStrTet
FSL S3-911	Typhimurium var. 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] "*"indicates intermediate resistance, i.e., Cro* indicates intermediate resistance to ceftriaxone

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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CHAPTER 3

MULTILOCUS SEQUENCE TYPING SUPPORTS THAT BOVINE AND HUMAN ASSOCIATED *SALMONELLA* REPRESENT DISTINCT AND OVERLAPPING POPULATIONS, INCLUDING CLONAL GROUPS WITH DUPLICATION AND DELETION EVENTS IN GENES ENCODING CELL SURFACE MOLECULE ASSOCIATED PROTEINS*

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ABSTRACT

A collection of 179 human and 166 bovine clinical *Salmonella* isolates obtained from across New York State over the course of one year were characterized using serotyping and multilocus sequence typing (MLST) scheme based on the sequencing of three genes (*fimA*, *manB*, and *mdh*). The 345 isolates were differentiated into 52 serotypes and 75 sequence types (STs). Serotypes and STs were not randomly distributed among human and bovine isolates and selected serotypes and STs were exclusively associated with human and bovine isolates. A number of common STs were geographically widely distributed, including isolates representing the emerging *Salmonella* serotype 4,5,12:i:-, which was found among human and bovine isolates in a number of counties in New York state. Phylogenetic analyses supported that serotype 4,5,12:i:- is closely related to *Salmonella* Typhimurium and that *Salmonella* Newport represents two distinct evolutionary lineages that differ in their frequency in human and bovine isolates. A number of isolates carried two copies of *manB* (48 isolates) or showed small deletion events in *fimA* (9 isolates). Overall, our data indicate that (i) serotyping and MLST typing both provide for sensitive subtype discrimination of *Salmonella*; (ii) bovine and human *Salmonella* subtypes represent distinct and overlapping populations; (iii) a number of *Salmonella* clonal groups, including emerging subtype 4,5,12:i:-, are geographically widespread among human and/or bovine populations; (iv) *Salmonella* Newport represents two distinct phylogenetic lineages that appear to be host specific; and (v) duplication and deletion events in *manB* and *fimA* may provide a mechanism for rapid diversification of *Salmonella* surface molecules.

INTRODUCTION

Salmonella is a Gram-negative pathogen found in a number of animals including birds, mammals, and reptiles. In the United States, *Salmonella* causes an

estimated 1.4 million food-borne illnesses a year (36) and salmonellosis accounts for over half of all food-borne outbreaks linked to bacterial pathogens (38). While *Salmonella* infections in developed countries are typically acquired through the consumption of contaminated food and water (7, 8, 11), direct contact with infected animals can also represent a source of human infections (9, 12, 31). While human non-typhoidal *Salmonella* infections generally manifest as gastroenteritis (37), systemic infection, which may require antibiotic treatment (49), can also occur.

Characterization of *Salmonella* isolates and human and animal salmonellosis surveillance have traditionally used serotyping for subtyping and strain differentiation (17). This technique relies upon the immunoreactivity of *Salmonella*'s O and H antigens. The O antigen is found in the lipopolysaccharide layer of the bacterial cell wall, while the H antigen is the filamentous portion of the flagella (17). While over 2,500 different *Salmonella* serotypes can be differentiated (17), some serotypes are commonly associated with human salmonellosis infections (e.g., serotypes Typhimurium, Enteritidis, Newport, Heidelberg; reference (17)), limiting the value of serotyping for human disease surveillance. More discriminatory subtyping methods, such as phage typing (3, 30) and pulsed-field gel electrophoresis (PFGE), are thus commonly used to subtype *Salmonella*, particularly as part of national and international salmonellosis surveillance systems (14, 15, 23, 24, 47). These methods are generally used in conjunction with serotyping, since serotyping information can still provide valuable information about host-associated subtypes, emergence of new subtypes, and historical trends on association of specific *Salmonella* subtypes with different hosts species. While serotyping, PFGE and phage typing, particularly if used in combination, can provide a high level of subtype discrimination, none of these methods provides appropriate information to infer phylogenetic relationships among *Salmonella* isolates and subtypes. Multilocus sequence typing (MLST) is a subtyping

method that determines the nucleotide sequences of full or partial housekeeping genes. Advantages of MLST not only include that the resulting DNA sequences data are non-ambiguous and easily compared between laboratories, e.g., through large WWW-base databases (23, 24, 47), but also that the DNA sequence data generated can be used to infer phylogenetic relationships among isolates, thus providing improved insight into the evolution and ecology of *Salmonella* subtypes. While MLST originally was defined as a sequencing-based subtyping approach that includes sequencing of 500 – 600 nt fragments for seven housekeeping genes (18, 48), the *Salmonella* MLST schemes described in the literature have used sequencing of three to four genes (35, 46); some of these MLST schemes also included sequencing of virulence or virulence associated genes, e.g., *spaM*, *fimA* (22, 46). While a 7-gene *Salmonella* MLST scheme is available through a WWW page maintained by the Max Plank Institute for infection biology in Berlin, Germany (6), we chose a previously described 3-gene MLST, which was shown to provide similar discriminatory power as a 7-gene MLST, for the study reported here, since sequencing of fewer genes provides for a more economical subtyping approach.

Transmission of *Salmonella* from animal populations to humans represents continuing concern (5), particularly since the prevalence of human salmonellosis cases does not appear to decrease despite considerable efforts to reduce foodborne diseases as specifically reported for the US (41). While a variety of animal reservoirs (e.g., poultry, pork, cattle, reptiles) can serve as direct or indirect, foodborne, sources for human *Salmonella* infections, our study reported here focused on probing the subtype diversity and epidemiology of human and bovine associated *Salmonella* populations. While source attribution of sporadic salmonellosis cases is difficult, a number of human salmonellosis outbreaks have been linked to contaminated foods of bovine origin (milk, beef) (38). In addition, it has been suggested that antibiotic use in dairy

cattle is selecting for multi-drug resistant *Salmonella*, which may be transmitted to the human population via food (4, 8) as supported by isolation of multi-drug resistant *Salmonella* from cattle (2, 34, 50) and human salmonellosis outbreaks linked to beef products contaminated with multi-drug resistant *Salmonella* (8, 10). Further, direct contact between cattle and humans may also be involved in the transmission of *Salmonella* between these populations (31, 45). We thus assembled a set of 345 human and bovine *Salmonella* isolated from the same general region (New York state and Vermont) over the same time (2004) for characterization using serotyping and a 3-gene MLST scheme in order to allow for meaningful and unbiased population-based comparisons of human and bovine-associated *Salmonella* subtypes and to probe the potential of bovine-associated subtypes to be transmitted to humans.

MATERIALS AND METHODS

***Salmonella* isolates**. A total of 345 *Salmonella* isolates, including 179 human and 166 bovine isolates were included in the study reported here (Supplemental Table S1, available at http://www.foodscience.cornell.edu/wiedmann/AlcaineTS1.doc). All isolates were obtained from clinical salmonellosis cases that occurred between January and December 2004. All human *Salmonella* isolates were received from the New York State Department of Health (NYSDOH). Specifically, for each month in 2004, 10 to 20 human clinical *Salmonella* isolates were randomly selected from all human clinical isolates received by NYSDOH that month for inclusion in our study. While the majority of human isolates (n=166) were obtained from patients residing in the counties outside the 5 New York city boroughs, a total of 11 isolates represented residents of New York city boroughs. The 166 bovine clinical *Salmonella* isolates were obtained from the Animal Health Diagnostic Center (AHDC) at Cornell University; isolates included in our study were obtained from specimens from cattle

with clinical salmonellosis symptoms, which represented either routine veterinary submissions (n=14) or submissions that were part of a prospective study on the frequency of clinical bovine salmonellosis (n=152). The prospective study enrolled 836 dairy herds in the Northeast U.S. Participating farms and veterinary practices were asked to submit fecal or tissue samples from dairy cattle with clinical signs consistent with salmonellosis to the AHDC for diagnostic testing. For enrolled herds, *Salmonella* culture and antimicrobial susceptibility testing along with diagnostic testing for other diseases with similar signs was paid for by the study. While the majority of bovine isolates (n=152) were obtained from farms located in New York, 16 isolates were obtained from farms in the neighboring state of Vermont. Bovine *Salmonella* isolates were selected so that only one isolate for each serotype isolated on a given farm from specimens collected on a given date in 2004 would be included. For a number of farms specimens collected at different dates in 2004 were submitted. In addition, for a number of farms multiple isolates with different serotypes were obtained from samples collected on a given day. Overall, animal isolates were obtained from a total of 64 different farms, including 56 and 8 located in New York and Vermont, respectively.

Human and bovine *Salmonella* isolates were serotyped at NYSDOH and the National Veterinary Services Laboratory (USDA-APHIS-VS, Ames, IA), respectively, using standard procedures (22).

PCR, DNA sequencing, and MLST typing. The MLST typing scheme used here was based on PCR amplification and sequencing of three genes (*manB*, *fimA*, and *mdh*) as previously reported (46). As also previously described (48), the full *fimA* ORF (558 nt) and partial *manB* and *mdh* ORFs (640 nt and 520 nt, respectively) were sequenced and used for allele assignments. While *Salmonella* lysates for PCR were initially prepared as previously described (48), preparation of purified *Salmonella*

DNA using the QIAamp DNA Mini Kit (Qiagen Inc., Chatsworth, CA) replaced this lysate protocol to yield more consistent PCR amplification.

PCR amplification of *manB*, *fimA*, and *mdh* was performed essentially as previously described (2). All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and quantified using either a Fluorescent DNA Quantitation Kit (Bio-Rad, Hercules, CA) or the Nanodrop spectophotometer (NanoDrop Technologies Inc., Willmington, DE). Purified PCR products were sequenced by the Biotechnology Resource Center at Cornell University or by Macrogen Inc. (Geumcheon-gu, Seoul, Korea) as previously described (2). All sequences were assembled and proofread using SeqMan, and aligned using the Clustal W algorithm in MegAlign (DNAStar, Madison, WI).

Allele assignments for individual genes were performed using DnaSP 4.0 (42); two sequences were assigned different allelic types if they differed by at least one nucleotide. STs were assigned so that isolates that have identical allelic types for all three genes have the same ST. Allelic types and STs were assigned to be consistent with previous studies that used the same MLST scheme (2, 46), i.e. ST 6 in the study reported here is identical to ST 6 reported by Alcaine et al. (2).

Phylogenetic analyses. Phylogenetic analyses were performed essentially as previously described (2). Briefly, maximum likelihood (ML) trees were constructed for each gene using one representative sequence for each allelic type for a given gene. MODELTEST (40) was used to find the most likely model of DNA substitution for a given gene and PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) was subsequently used to construct ML trees using the appropriate substitution model (TrNef+G for *fimA* and *mdh*; HKY+I+G for *manB*). Each tree was rooted with the *manB*, *fimA*, or *mdh* sequence for *E. coli* O157:H7 (29), which served as the outgroup. Phylogenetic trees were only constructed based on individual sequences and not based

on concatenated sequences, since a number of *manB* sequences showed mixed bases due to the presence of two gene copies (48), preventing their inclusion in phylogenetic analyses.

Statistical analyses. The frequency distributions of serotypes and sequence types between human and bovine isolates were compared using a $?^2$ test of independence. For comparisons where one or more of the expected values were <5, Fisher's exact test was performed. P-values <0.05 were considered statistically significant. Chi-Square and Fisher's Exact tests were performed using the free statistical software SISA-Tables (available at http://www.quantitativeskills.com/downloads/) or SAS version 9.1 (SAS Institute Inc., Cary, NC). P-values for Exact tests for large contingency tables (e.g., 2 x 10) were determined using a Monte Carlo simulations in SAS version 9.1.

Simpson's Index of Discrimination (SID) was calculated as described by Hunter et al. (32); this index provides an indication of the discriminatory power of a given subtyping method (32) as well as an estimate of the subtype diversity within a given population (25). The 95% confidence intervals for the SID were calculated as described by Grundmann et al. (26). All calculations were performed using Microsoft Excel (Microsoft, Seattle, WA).

Since, among the bovine *Salmonella* isolates included in this study, multiple isolates obtained from the same farm at different sampling times showed the same serotypes and STs, indicating re-isolation of a persistent subtype on a given farm, only one isolate representing each unique serotype/ST combination was included in the $?^2$ test and SID calculations to avoid over representation of a subtype due to resampling.

Access to detailed isolate information. All isolate information for this study, including 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 linked to the reference for this manuscript and are listed at http://cbsusrv01.tc.cornell.edu/users/PathogenTracker/pt2/search/display_list.aspx?refid=260.

RESULTS AND DISCUSSION

A total of 345 human and bovine *Salmonella* isolated from clinical cases that occurred in New York and a small neighboring state (Vermont) were characterized by serotyping and MLST targeting three selected genes in order to better understand the ecology and epidemiology of human and bovine associated *Salmonella*. Detailed analysis of serotypes and MLST data indicates that (i) serotyping and MLST typing both provide for sensitive subtype discrimination of *Salmonella*; (ii) bovine and human *Salmonella* subtypes represent distinct and overlapping populations; (iii) a number of *Salmonella* clonal groups, including emerging subtype 4,5,12:i:-, are geographically widespread among human and/or bovine populations; (iv) *Salmonella* Newport represents two distinct phylogenetic lineages that appear to be host specific; and (v) duplication and deletion events in *manB* and *fimA* may provide a mechanism for rapid diversification of *Salmonella* surface molecules.

Serotyping and MLST typing both provide for sensitive subtype discrimination of *Salmonella.* The 345 *Salmonella* isolates included in this study could be differentiated into a total of 52 serotypes and 75 sequence types (STs). *fimA* and *mdh* sequence data allowed differentiation of 45 and 29 allelic types. Analysis of *manB* sequence data revealed a total of 48 isolates that showed reproducible double peaks at 1 to 15 nt positions, consistent with the presence of two copies of *manB* in these isolates as previously reported for a smaller number of isolates (2). For each *manB* nt position with a double peak an International Union of Pure and Applied Chemistry

(IUPAC) ambiguity nt code indicating the presence of the two bases found (e.g., Y indicates presence of C or T) was used to designate the final sequences for a given isolates. Isolates were assigned different *manB* allelic types if two isolates differed in their nt sequence, including the ambiguous nucleotides (mixed bases), consistent with similar approaches that have been used to assign subtypes based on 16S rDNA sequence data that indicated the presence of multiple distinct 16S rDNAs in a given organism (43). Using this approach a total of 55 *manB* allelic types were differentiated.

When serotype and MLST typing data were combined to assign overall subtypes (i.e., only isolates with the same serotype and ST were considered the same subtype) a total of 84 subtypes were differentiated. Analysis of combined serotype/ST-based subtypes allowed us to analyze the *Salmonella* diversity on the 20 farms for which *Salmonella* isolates were collected over multiple visits in 2004 (Table 3.1). Interestingly, on a number of farms a given *Salmonella* subtype persisted over time, e.g., on one farm a ST11 *Salmonella* Newport strain was isolated from samples collected over 28 separate visits (Table 3.1). These data not only show persistence of specific *Salmonella* subtypes over time in cattle in a given farm, but also allowed us to assure that only one isolate representing each unique serotype/ST combination found on a given farm was included in the subsequent analyses reported below to avoid over representation of a subtype due to resampling, yielding a total of 81 unique bovine isolates.

A total of 17 serotypes included two or more STs, including differentiation of 7 and 5 STs within serotype Newport and Typhimurium isolates. Among the 75 STs differentiated in this study, 7 included isolates representing multiple serotypes. These finding are consistent with previous studies, which also found differentiation of multiple serotypes within a given (2, 46). Overall discriminatory ability, as

TABLE 3.1 Farms with multiple sample-submission dates resulting in *Salmonella* isolation from clinically infected animals

Farm ID	No. of farm sample dates with positive *Salmonella* samples	Subtypes isolated (no. of isolates)
510	30	ST11/Newport (28); ST85/Newport (1); ST6/4,5,12:i:- (1)
261	24	ST6, 4,5,12:i:- (18); ST17/Kentucky (4); ST34/Kentucky (1); ST6/Typhimurium (1)
223	15	ST60/Infantis (15)
329	5	ST9/Montevideo (1); ST44/Muenster (3); ST62/Thompson (1)
186	5	ST75/Adelaide (1); ST8/Typhimurium (2); ST8/T.Copenhagen (2)
524	5	ST6/4,5,12:i:- (1); ST11/Newport (4)
152	4	ST11/Newport (4)
490	4	ST11/Newport (4)
163	3	ST60/Infantis (1); ST11/Newport (2)
259	3	ST44/Muenster (3)
488	3	ST11/Bardo (1); ST11/Newport (3)
584	3	ST2/Agona (2); ST6/Typhimurium (1)
97	2	ST8/T. Copenhagen (2)
105	2	ST11/Newport (1); ST8/Typhimurium (1)
125	2	ST6/Typhimurium (2)
208	2	ST6/Typhimurium (2)
303	2	ST11/Newport (2)
320	2	ST11/Newport (2)
415	2	ST9/Montevideo (1); ST6/Typhimurium (1)
764	2	ST6/Typhimurium (1); ST49/Typhimurium (1)

determined by SID, for serotyping and MLST was 0.918 and 0.922 respectively. When serotype and MLST typing data were combined to assign overall subtypes a total of 84 subtypes were differentiated, with an overall SID of 0.945. While other subtyping methods, in particular PFGE, appear to provide for even more sensitive subtype discrimination (23), we conclude that serotyping and MLST provide for appropriate subtype discrimination to probe the ecology and epidemiology of human and bovine *Salmonella*, particularly since MLST data allow for phylogenetic analysis and definition of *Salmonella* clonal groups (46).

Bovine and human *Salmonella* subtypes represent distinct and overlapping populations. The *Salmonella* isolates characterized represented 35 and 6 serotypes unique to human and bovine isolates, respectively, as well as 11 serotypes found among both host species. The most common human associated serotypes were Typhimurium; Enteritidis; Newport; 4,5,12:i:-; and Heidelberg, largely consistent with 2003 CDC data, which listed serotypes Typhimurium, Enteritidis, Newport, Heidelberg and Javiana as the five most commonly reported human *Salmonella* serotypes in the United States (17), supporting that the human isolates included in our study are representative of human clinical disease associated subtypes in the US. Comparison of the frequency distributions of serotypes among human and bovine isolates using an overall chi-square analysis (with all serotypes that occurred =4 times grouped into a single category, termed "rare serotypes") showed that serotypes were not randomly distributed among human and bovine isolates ($p<0.001$; Monte Carlo simulation of Fisher's exact test). Individual 2x2 chi-square tests (comparing the frequency of a given serotypes to all other serotypes) showed that serotypes Enteritidis, Heidelberg, and 4,5,12,i:- were overrepresented among human isolates while serotypes Newport and Muenster were overrepresented among bovine isolates. Serotypes Enteritidis and Heidelberg were actually exclusive to human isolates,

consistent with their well-recognized association with poultry and particularly chickens (17, 26, 44) and rare presence in cattle (17, 26). In addition to being common among human isolates, serotypes Typhimurium and Newport were also the two most common bovine associated serotypes, consistent with US-wide CDC prevalence data (17) and consistent with the fact that human salmonellosis outbreaks caused by these two serotypes have been linked to the consumption of contaminated beef and dairy products (7, 8, 39). While this provides initial evidence that cattle may be an important source of human infections caused by these two serotypes, the sources of human *Salmonella* Newport infections may likely differ dependent on the specific *Salmonella* Newport clonal group as discussed below. In addition to the chi-square test data, which showed that the individual serotype frequency differed among bovine and human isolates, SIDs also showed that human serotypes diversity (SID = 0.931 ± 0.019) was significantly higher than bovine serotype diversity (SID = 0.834 ± 0.056), supporting that distinct *Salmonella* populations are associated with these two host species.

MLST identified 57 and 8 STs that were unique to human and bovine isolates, respectively, as well as 10 STs that were found in both host species. Comparison of the frequency distributions of STs among human and bovine isolates using an overall chi-square analysis (with all STs that occurred =4 times grouped into a single category, termed "rare STs") showed that STs were not randomly distributed among human and bovine isolates ($p<0.001$; Monte Carlo simulation of Fisher's exact test). Individual 2x2 chi-square tests (comparing the frequency of a given ST to all other STs) showed that two and three STs were overrepresented among human and bovine isolates, respectively, further supporting that human and bovine isolates represent distinct subtype populations. ST3 and ST 14, which were overrepresented among human isolates represent serotypes Heidelberg and 4,12:r:- (ST3) and Enteritidis (ST

14), consistent with serotypes results discussed above. Interestingly ST 8, which represented serotype Typhimurium and related serotypes, was overrepresented among bovine isolates and never isolated from human clinical cases, potentially indicating that this specific ST shows bovine host specificity. ST 11 and ST 44, which were also more common among bovine than human isolates, represented serotypes Newport and Bardo (ST11) and Muenster (ST44), consistent with overrepresentation of these serotypes among bovine isolates (Table 3.2). While ST data thus largely confirmed the host association of specific serotypes, they also identified a bovine associated ST within serotype Typhimurium, which could not have been identified based on serotype data alone.

SID also showed that human ST diversity (SID = 0.938 ± 0.023) was significantly higher than bovine ST diversity (SID = 0.819 ± 0.057), providing additional support human and bovine associated *Salmonella* represent distinct populations. The higher ST and serotype diversity found among human *Salmonella* isolates likely reflects the fact that human *Salmonella* infections can originate from a number of distinct source populations and reservoirs (e.g., avian, reptile, bovine, porcine) and indicates that a large number of subtypes found in these different hosts have the ability to cause human disease.

A number of *Salmonella* clonal groups, including emerging subtype 4,5,12:i:-, are geographically widespread among human and/or bovine populations. Analysis of geographic source data of human and bovine isolates (Table 3.3) showed that a number of STs are distributed widely across New York State, including STs 3, 14, and 36, which were only found among human isolates, and ST8, which was only found among human isolates. ST 6 and ST 11, the two most common STs found in this study, were widely distributed among both human and bovine populations in New York State. Interestingly, ST 6 not only contains *Salmonella* serotype Typhimurium

TABLE 3.2 Distribution of *Salmonella* serotypes among sequence types (STs) and human and bovine isolates

Serotype[a]	ST	No. of isolates from	
		Human	Cattle[b]
4,5,12:i:-*	6	12	3
Agona	1, 2	3	4
Enteritidis***	14, 36	26	0
Heidelberg*	3, 26, 50	10	0
Mbandaka	64, 65, 73	4	1
Montevideo	9, 56, 57, 67	4	3
Muenster*	44	1	5
Newport***	11, 13, 33, 46, 76, 78, 85	18	26
Saintpaul	38, 81	5	0
Thompson	43, 62	5	3
Typhimurium	6, 7, 8, 47, 49	30	20
Urbana	52	5	0

[a]Only serotypes that occurred = 5 are listed separately (for animal isolates only one isolate with a given serotype and ST per farm was counted). Additional serotypes that occurred =4 timesinclude (number of human and unique bovine isolates per farm are included in parenthesis and marked as "h" or "b"): 4,12:i:- (1b); Abony (2h); Adelaide (1h, 1b); Agbeni (1h); Anatum (3h); Arechavaleta (1h); 4,12:r:- (2h); Bardo (2b); Berta (2h); Blockley (1h); Braenderup (1h); 1,7:-:1,5 (3h); Cubana (1h); Dublin (2h); Give (1h); Hadar (2h); Hartford (1h); Havana (1b); Infantis (2h, 2b); Javiana (4h); Kentucky (3b); Kintambo (1h); Litchfield (1h); Muenchen (3h); Nyanza (1h); Oranienburg (1h, 1b); Panama (3h); Paratyphi B (1h); Paratyphi B var. Java (2h); Paratyphi C (1h); Pomona (1h); Poona (2h); Rough o:i:1,2 (1b); Rublislaw (1h); Schwarzengrund (3h); Senftenberg (1h); Stanley (2h); Typhimurium var. Copenhagen (4b); Weltvreden (1h); Worthington (1h).

Serotypes which differ significantly in frequency among human and animal isolates, as determined by Chi square test or Fisher's exact test, are marked with * ($p<0.05$), ** ($p<0.01$), or *** ($p<0.001$).

[b]Only one isolate representing each unique serotype/ST combination found on a given farm was counted; these numbers were used to avoid over representation of a subtype due to resampling on a given farm (e.g., when isolates with the same serotype/ST combination were isolated on different dates on the same farm; see Table 3.1).

TABLE 3.3 Distribution of *Salmonella* sequence types (STs) among human and bovine isolates and among different counties in New York State

ST[a]	Serotype	No of isolates from		County origin of isolates from (no. of isolates)[c]	
		Humans	Cattle[b]	Humans	Cattle[b]
3*	Heidelberg; 4,12:r:-	10	0	Dut (1); Eri (1); Fra (1); Mon (1); Nas (2); New (1); One (1); Suf (2)	None
6	Typhimurium; 4,12:i:-; 4,5,12:i:-	37	20	Alb (1); Brx (1); Cha (1); Cmg (1); Cor (1); Dut (1); Eri (3); Fra (1); Kin (1); Mon (1); Nas (4); Ono (2); Ora (1); Ots (1); Stl (1); Ste (2); Suf (7); Tom (1); Was (2); Wes (4)	Cat (1); Cli (3); Cor (1); Gen (1); Nia (1); Ont (1); Orl (1); Ren (3); Stl (1); Tom (1); Was (1); Wyo (5)
8***	Typhimurium; Rough o:i:1,2; T. var Copenhagen	0	7	None	Cay (1); Cno (1); Osw (2); Was (3)
11***	Newport; Bardo	9	27	Cno (1); Nia (1); Put (1); Sar (1); Suf (1); Uls (1); Unk (1); Way (1); Wes (1)	Cay (1); Cno (1); Cvt (2); Cli (2); Cor (1); Eri (1); Fvt (6); Gen (1); Lvt (1); Lew (1); Nia (3); One (2); Ono (1); Sen (1); Stl (1); Wyo (2)

[a]The p-values of sequence types (ST) which differ significantly in frequency among human and animal isolates, as determined by Chi square test or Fisher's exact test, are indicated as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

[b]County codes are: Alb Albany, Brx Bronx, Brm Broome, Cat Cattaraugus, Cay Cayuga, Cha Chautauqua, Cmg Chemung, Cno Chenango, Cvt Chittenden VT, Cli Clinton, Cor Cortland, Del Delaware, Dut Dutchess, Eri Erie, Fra Franklin, Fvt Franklin VT, Gen Genesee, Kin Kings, Lvt Lamoille VT, Lew Lewis, Liv Livingston, Mon Monroe, Nas Nassau, New New York, Nia Niagra, One Oneida, Ono Onondaga, Ont Ontario, Ora Orange, Orl Orleans, Osw Oswego, Ots Otsego, Put Putnam, Ren Rensselaer, Sar Saratoga, Sch Schenectady, Sen Seneca, Stl St. Lawrence, Ste Steuben, Suf Suffolk, Tom Tompkins, Uls Ulster, Unk Unkown, Was Washington, Way Wayne, Wes Westchester, Wyo Wyoming

[c]Rare indicates sequence types with fewer than 5 isolates, there were 64 such sequence types

[d]NS Not Shown

TABLE 3.3. (Continued)

ST[a]	Serotype	No of isolates from		County origin of isolates from (no. of isolates)[c]	
		Humans	Cattle[b]	Humans	Cattle[b]
14**	Enteritidis	19	0	Brm (1); Dut (1); Eri (3); Kin (1); Mon (2); Nas (3); Ono (2); Ora (1); Suf (2); Unk (1); Wes (2)	None
36	Enteritidis	7	0	Alb (1); Del (1); Eri (1); Mon (1); Nas (1); Tom (1); Wes (1)	None
44*	Muenster	1	5	Cmg (1)	Fra (1); Liv (3); Orl (1); Wyo (2)
52	Urbana	5	0	Cli (2), Sch (3)	None
62	Thompson	4	2	Nas (1), Sar (1), Uls (1), Way (1)	Liv (1), Nia (1)
Rare***[c]	Various	87	20	NS[d]	NS[d]

[a]The p-values of sequence types (ST) which differ significantly in frequency among human and animal isolates, as determined by Chi square test or Fisher's exact test, are indicated as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

[b]County codes are: Alb Albany, Brx Bronx, Brm Broome, Cat Cattaraugus, Cay Cayuga, Cha Chautauqua, Cmg Chemung, Cno Chenango, Cvt Chittenden VT, Cli Clinton, Cor Cortland, Del Delaware, Dut Dutchess, Eri Erie, Fra Franklin, Fvt Franklin VT, Gen Genesee, Kin Kings, Lvt Lamoille VT, Lew Lewis, Liv Livingston, Mon Monroe, Nas Nassau, New New York, Nia Niagra, One Oneida, Ono Onondaga, Ont Ontario, Ora Orange, Orl Orleans, Osw Oswego, Ots Otsego, Put Putnam, Ren Rensselaer, Sar Saratoga, Sch Schenectady, Sen Seneca, Stl St. Lawrence, Ste Steuben, Suf Suffolk, Tom Tompkins, Uls Ulster, Unk Unkown, Was Washington, Way Wayne, Wes Westchester, Wyo Wyoming

[c]Rare indicates sequence types with fewer than 5 isolates, there were 64 such sequence types

[d]NS Not Shown

isolates, but also includes *Salmonella* serotypes 4,5,12:i:-; and 4,12:i:- (Table 3.3), indicating that these serotypes share a common ancestor, consistent with phylogenetic trees (Fig. 3.1), which grouped ST6 with other *Salmonella* Typhimurium STs (ST 7, 8, 47, 49). In addition, two preliminary PFGE, RAPD, plasmid profile, ribotyping-based studies on small isolate sets (<50 isolates) also previously concluded that serotype 4,5,12:i:- originated from a *Salmonella* Typhimurium ancestor (19, 27). The ecology and epidemiology of *Salmonella* serotype 4,5,12:i:- is of particular interest since it appears to represent an emerging human-disease associated *Salmonella* serotype (17). In 2003, serotype 4,5,12:i:- was identified as 14th most common human *Salmonella* serotype in the US (17), but due to difficulties in the classification of this serotype many 4,5,12:i:- isolates have simply been reported as Subspecies I, Group B, so its prevalence may have been greater than reported (17). Serotype 4,5,12:i:- was also implicated in a food-borne salmonellosis outbreak in New York city in 2002 (1). The observation that this serotype was the 4th most common human associated serotype in our study and that ST6, which includes 4,5,12:i:- as well as other related serotypes (Table 3.3), is the most common human disease and 2nd most common bovine associated serotype indicates the public health importance of this clonal group, including the potential importance of bovine hosts as a reservoir for this clonal group.

***Salmonella* Newport represents two distinct phylogenetic lineages that appear to be host specific.** MLST data showed that *Salmonella* serotype Newport isolates represented seven STs (Table 3.2). Phylogenetic trees based on the sequenced regions of *fimA* (Fig. 3.1a) and *mdh* (Fig. 3.1b) revealed the serotype Newport STs represented two distinct clonal groups, consistent with preliminary data for three bovine and two avian Newport isolates (46). Based on the larger dataset (86 Newport isolates) reported here, we have designated these two lineages as *Salmonella* Newport type A and B. The observation that a single Newport type A ST (ST85) clusters separate

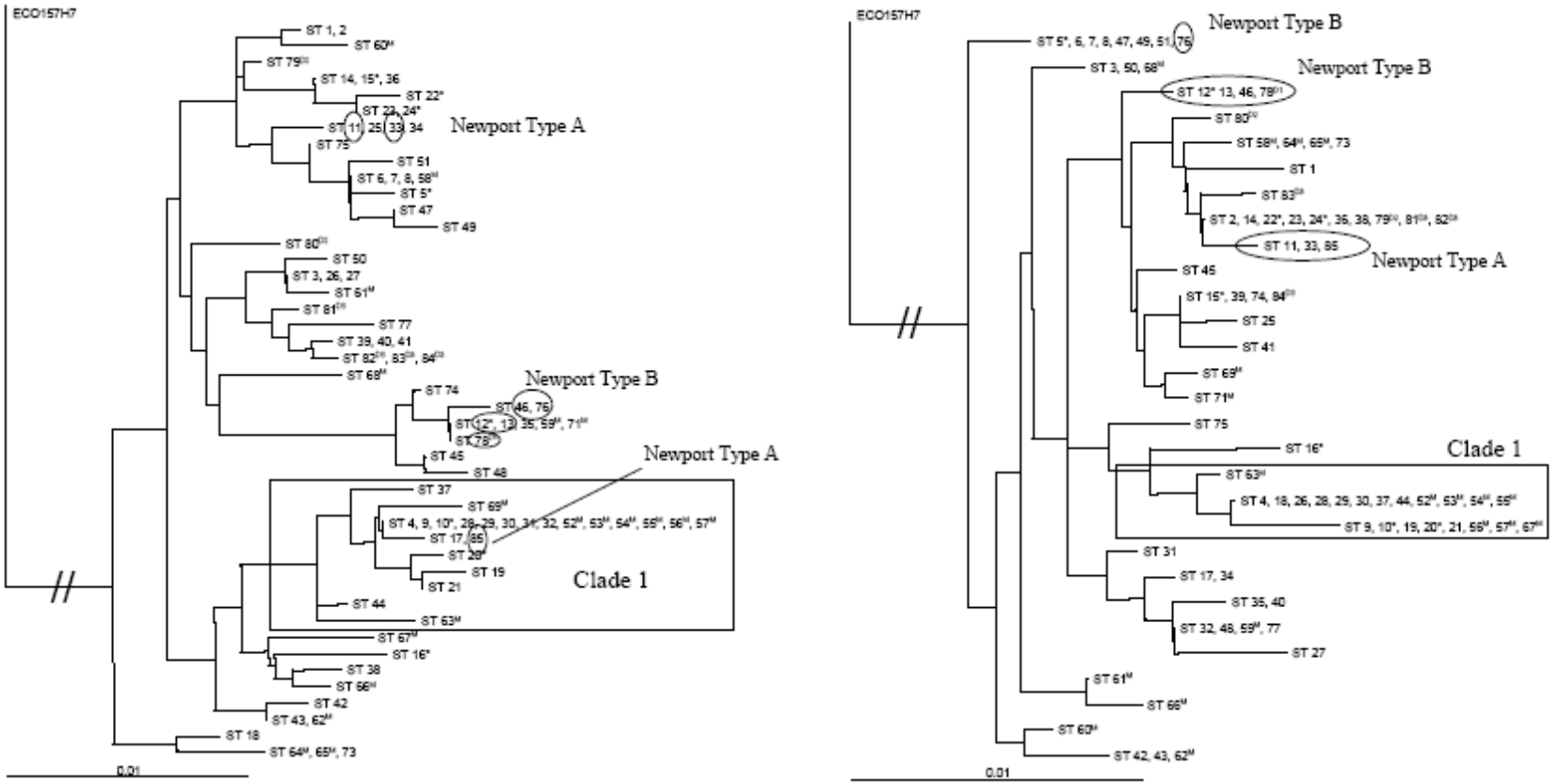

FIGURE 3.1. Phylogenetic trees based on *Salmonella fimA* (A) and *mdh* (B) gene sequences. Maximum likelihood trees were built using PAUP* and one representative isolate for each sequence type, including STs previously described by Sukhnanand et al. (48), which were not represented among the isolates found here (these isolates are indicated by a *). ^{M} indicates STs representing isolates that contain a *manB* gene duplication; the branch labeled as "clade 1" represents a number of related STs, including STs (52 – 57, 63, 67) that correspond to isolates with two *manB* copies, indicating a common ancestral event that was responsible for the two *manB* genes found in these isolates. $^{D1, D2, D3}$ indicate STs representing isolates that contain *fimA* deletion types D1 through D3 (see Fig. 2). STs representing the two distinct *Salmonella* Newport lineages (designated as type A and B) are marked by circles and the respective type (A or B).

from other type A isolates in the *fimA* tree (Fig. 3.1a) and that a single Newport type B ST (ST76) clusters separate from other type B isolates in the *manB* tree (Fig 3.1b) likely represents horizontal gene transfer events of *fimA* and *manB* alleles, respectively, and does not affect this conclusion, since these STs group with isolates the same type (A or B) in the other trees. Overall, Newport type A represented three STs (11, 33, and 85) and includes 11 human isolates and 68 isolates from different cattle farms, consistent with previous data that ST 11 was associated with bovine sources (46). *Salmonella* Newport type B, represented four STs (13, 46, 76, and 78), all isolates of this type (n=7) were obtained from human clinical cases. Statistical analysis (chi-square test) indicated that the frequency distribution of human and bovine isolates among these two *Salmonella* Newport types differs significantly ($p<0.001$), indicating that these types differ in their host association. Since a previous study has shown *Salmonella* Newport isolates of avian origin grouped into type B (46), we hypothesize that *Salmonella* Newport types A and B represents two bovine and avian associated lineages that can both be transmitted to humans. Interestingly, antimicrobial resistance data showed that all seven Newport type B isolates were sensitive to all commonly used antibiotics, while eight of of the 14 tested type A isolates showed a multidrug resistance (MDR) phenotype with a ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline (ACSSuT) resistance type (Richards, Alcaine, McDonough, and Wiedmann, unpublished data), indicating that MDR *Salmonella* Newport, an emerging pathogen of public health relevance (17, 20, 28), represents a bovine associated lineage. MLST typing of human Newport isolates may thus not only provide a means for linking human salmonellosis cases or outbreaks to likely food sources, particularly since human outbreaks of *Salmonella* serotype Newport infections have been linked to both beef (8) and poultry sources

(13), but also may be critical for monitoring the sources and spread of MDR *Salmonella* Newport.

Duplication and deletion events in *manB* and *fimA* may provide a mechanism for rapid diversification of *Salmonella* surface molecules. DNA sequence data for *manB* revealed the presence of two copies of *manB* in a total of 27 human and 21 bovine isolates, as indicated by the presence of mixed bases at a number of nt positions after sequencing of PCR products. These findings are consistent with results from a previous study where we reported and confirmed, using cloning and sequencing of *manB* PCR products, the presence of two *manB* genes in 3 *Salmonella* serotype Montevideo isolates (46). Phylogenetic analyses of the duplicate *manB* genes found in these serotype Montevideo isolates indicated that an additional *manB* copy had likely been introduced into an ancestral strain by horizontal gene transfer from a different *Salmonella* serotype similar to serotype Javiana (41). In the study reported here, we found evidence for the presence of two *manB* genes in 16 *Salmonella* serotypes (including Montevideo) and in 19 STs (Table 3.4). *fimA* and *mdh*-based phylogenetic trees (Fig. 3.1) showed that STs representing isolates with two *manB* copies represent a number of distinct subtypes, indicating that multiple independent horizontal gene transfer events (or duplication events) contributed to the presence of two *manB* copies in different *Salmonella* subtypes, indicating positive selection for the presence of multiple *manB* copies. Interestingly, a number of STs (52 – 57, 63, 67), which represent isolates with two *manB* copies, cluster together (designated as clade 1 in Fig. 3.1), indicating a common ancestral event that was responsible for the two *manB* genes found in these isolates. While the benefit of two distinct *manB* genes in a single isolates remains to be determined, the fact that *manB* encodes for phosphomannomutase, an enzyme that is part of the chemical pathway necessary to produce GDP-D-mannose, an important sugar subunit of the *Salmonella*'s

TABLE 3.4 Serotypes that include at least one isolate that carries two *manB* copies[a]

Serotype	Sequence types of human isolates (no of isolates) within a given serotype that carry		Sequence types of cattle isolates (no of isolates) within a given serotype that carry[b]	
	two *manB* copies	one *manB* copy	two *manB* copies	one *manB* copy
Agbeni	63 (1)	none	none	none
Braenderup	61 (1)	none	none	none
1,7:-:1,5	58 (1)	42 (1), 43 (1)	none	none
Cubana	71 (1)	none	none	none
Havana	none	none	69 (1)	none
Infantis	60 (2)	none	60 (2)	none
Kintambo	59 (1)	none	none	none
Mbandaka	64 (2)	73 (2)	65 (1)	none
Montevideo	56 (2), 57 (1), 67 (1)	none	none	9 (3)
Nyanza	66 (1)	none	none	none
Oranienburg	53 (1)	none	53 (1)	none
Poona	55 (1)	28 (1)	none	none
Rublislaw	54 (1)	none	none	None
Thompson	62 (4)	43 (1)	62 (2)	43 (1)
Urbana	52 (5)	none	none	None
Worthington	68 (1)	none	none	None

[a]Serotypes for which all isolates carry only a single *manB* gene are not included in this table

[b]Only one isolate representing each unique serotype/ST combination found on a given farm was counted; these numbers were used to avoid over representation of a subtype due to resampling on a given farm (e.g., when isolates with the same serotype/ST combination were isolated on different dates on the same farm; see Table 3.1)

O antigen (33), may suggest that the presence of two *manB* genes provides a possible mechanism to rapidly generate serotype diversity. This is consistent with the observation that isolates in clade 1 that carry two *manB* copies represent a total of 6 serotypes, including Agbeni, Montevideo, Nyanza, Oranienburg, Rublislaw, and Urbana. Further research is clearly needed to better understand potential contributions of multiple *manB* copies in generating *Salmonella* serotype diversity.

Interestingly, *fimA* sequence analysis revealed three different deletions in the 3' coding region of *fimA* (Table 3.5 and Fig. 3.2), including a 5 bp deletion (deletion type D1), which leads to a premature stop codon and is only found in the three ST 78 isolates. Deletion types D2 and D3 on the other hand are in frame 3 bp deletions that resulted in the loss of an alanine and threonine in the respective protein sequences (Fig. 3.2). A deletion similar to D3, was also reported in *Salmonella* serotype Typhi (16). A *fimA* based phylogeny (Fig. 3.1a) showed that sequences with a deletion types D1 and D3 each form a separate clusters, indicating that each of these deletions represents one event with a common ancestors. The two STs carrying deletion type D2 clustered separately in the *fimA* tree, possibly indicating two separate deletion events or horizontal transfer of a *fimA* fragment carrying this deletion among distinct *Salmonella* subtypes. Interestingly, in the *mdh* tree some STs representing deletion type D3 clustered represented three distinct *mdh* lineages; a *manB* phylogenetic tree (not shown) also further supported that some STs with same *fimA* deletion represent distinct clades, supporting possible horizontal gene transfer of *fimA* genes with deletion type D3 between unrelated *Salmonella* subtypes. While *fimA* encodes for FimA, the major shaft subunit of type 1 fimbriae in *Salmonella enterica*, the second subunit of type 1 fimbriae, FimH, is responsible for the binding of the fimbriae to specific sugars. Binding specificity of type 1 fimbriae has been proposed though to be largely due to the interaction between the FimA and FimH subunits, rather than due to

TABLE 3.5 Serotypes that include at least one isolate that carries a deletion in the 3' end of *fimA*[a]

Serotype	*fimA* deletion type	Sequence types of human isolates (no of isolates) within a given serotype that carry		Sequence types of cattle isolates (no of isolates) within a given serotype that carry[b]	
		a *fimA* deletion	no *fimA* deletion	a *fimA* deletion	no *fimA* deletion
Newport	D1	78 (3)	11 (9), 13 (1), 33 (2), 46 (2), 76 (1), 78 (1)	none	11 (25), 85 (1)
Weltvreden	D2	79 (1)	none	none	none
Paratyphi C	D2	80 (1)	none	none	none
Saint Paul	D3	81 (1)	38 (4)	none	none
Berta	D3	82 (1), 83 (1)	none	none	none
Stanley	D3	84 (1)	39 (1)	none	none

[a]Serotypes for which all isolates carry no deletion in the 3' end of *fimA* are not included in this table

[b]Only one isolate representing each unique serotype/ST combination found on a given farm was counted; these numbers were used to avoid over representation of a subtype due to resampling on a given farm (e.g., when isolates with the same serotype/ST combination were isolated on different dates on the same farm; see Table 3.1)

A

Deletion Types	DNA Sequence
Wild Type	GCACGCTATAAGGCAACCGCCGCCGCCACGACGCCAGGCCAG GCTAAT
d1	GCACGCTATAAGGCAACCGCCGCCGCCGCGACGCCAGGC------TAAT
d2	GCACGCTATAAGGCAACCGCCGCC------ACGACGCCAGGCCAGGCTAAT
d3	GCACGCTATAAGGCAACCGCCGCCGCG------ACGCCAGGCCAGGCTAAT

B.

Deletion Types	Amino Acid Sequence
Wild Type	ARYKATAAATTPGQANADATFIMKYE.
d1	ARYKATAAAATPG.
d2	ARYKATAA-TTPGQANADATFIMKYE.
d3	ARYKATAAA-TPGQANADATFIMKYE.

FIGURE 3.2. DNA and amino acid sequences of *fimA* deletion types d1, d2, and d3. (A) nt sequence surrounding the deletions in the 3' region of *fimA*. (B) aa sequence surrounding the deletions in the 3' region of *fimA;* first aa corresponds to the first codon shown in (A). While the aa sequence shown includes the stop codon, the nt sequence shown in (A) is missing the nucleotides encoding the last 10 aa

the primary structure of the FimH subunit alone (21), suggesting that variations in the shaft subunits, e.g., due to deletion events, could lead to changes in sugar specificity, and thus changes in binding. While functional studies are needed to characterize the phenotypic importance of the *fimA* deletion described here, the occurrence of multiple independent deletion events and their apparent fixation in different lineages after horizontal gene transfer indicates potential selective advantages associated with these deletions, such as changes in the specificity or avidity of fimbrial binding to host cell sugars. In this context it is interesting to note that the *fimA* deletions were only found in *Salmonella* isolates from human sources, potentially indicating a selection for these deletions in specific hosts.

CONCLUSIONS

The data reported here show that combined use of serotyping with MLST and phylogenetic analyses can provide important insight into the evolution, ecology and epidemiology of *Salmonella* subtypes associated with different host species, which cannot be achieved with traditional subtyping methods that do not allow for phylogenetic analyses. Our results specifically support that human and bovine *Salmonella* isolates represent separate, but overlapping populations that include widely distributed subtypes found in both host populations (e.g., the emerging subtype 4,5,12:i:-) as well as host specific subtypes, including host-specific clonal groups within a given serotype (e.g., ST8, a bovine associated ST, which includes serotype Typhimurium isolates). Definition of two distinct *Salmonella* Newport lineages, including one bovine associated lineage that also includes MDR *Salmonella* Newport, in particular provides an opportunity to monitor emergence, transmission, and spread of these MDR strains in different host populations. Definition of host-specific and host associated subtypes will not only improve our understanding of the biology of this important mammalian pathogen, but also will enhance our ability for accurate

source tracking of human salmonellosis cases and outbreaks.

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