GENOTYPIC AND ANTIMICROBIAL RESISTANCE TRENDS IN SALMONELLA ON MICHIGAN DAIRY FARMS

By

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ABSTRACT

GENOTYPIC AND ANTIMICROBIAL RESISTANCE TRENDS IN *SALMONELLA* ON MICHIGAN DAIRY FARMS

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The health and welfare of cattle and humans are inextricably linked, and the momentum behind the “One Health” paradigm in veterinary and human medicine continues to grow. The linkage of livestock and human health is particularly true for zoonotic *Salmonella* that contaminate the human food supply. *Salmonella* causes the largest number of foodborne deaths and hospitalizations in the United States; livestock, including dairy farms, are a primary reservoir for those infections. Increases in the antimicrobial resistance and incidence of salmonellosis in humans have been associated with the emergence of novel *Salmonella* strains in livestock. Therefore, improved knowledge of the frequency and drivers for changes in the population of *Salmonella* on livestock farms could lead to positive impacts on public health.

This study used a long-term longitudinal approach to assess changes in the prevalence, antimicrobial resistance, and genetic subtypes of *Salmonella* on Michigan dairy farms. The overall goal was to determine genotypic population changes that were associated with changes in the prevalence and antimicrobial resistance of *Salmonella* within farms. Specifically, this study addressed the following four objectives: 1) Determine within-farm changes in the antimicrobial susceptibility of *Salmonella* between 2000-2001 and 2009 2) Determine within-farm changes in the prevalence of *Salmonella* using seasonally-matched sampling visits in 2000-2001 and 2009 3) Identify the serotypes, sequence types, and PFGE banding patterns for *Salmonella* recovered in each

time period 4) Determine the association between antimicrobial susceptibility changes and changes in the population of molecular subtypes.

Results from this study suggest that the overall prevalence of *Salmonella* on Michigan dairy farms has increased between 2000-2001 and 2009. Increases in *Salmonella* prevalence were associated with increases in herd size and changes in management practices. The proportion of isolates that were resistant to multiple antimicrobials was less in 2009 relative to 2000-2001. Additionally, there were decreases in the minimum inhibitory concentrations (MICs) for nine of the 15 tested antimicrobials. Results of the sequence typing and PFGE profiles show an overall high relatedness, and long-term persistence of *Salmonella* within farms. Analysis of the antimicrobial resistance changes and genotypic changes suggest that downward shifts in MICs were associated with a shift in the population favoring serogroup C1. Additionally, recovery of MDR strains in 2000-2001, and susceptible strains of the same serotype in 2009 suggest displacement of MDR subtypes by susceptible subtypes.

This Dissertation is dedicated to Ella, Gracie, and Sammy

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ABBREVIATIONS

Name	Abbreviation
Multilocus sequence typing	MLST
Pulsed-field gel electrophoresis	PFGE
Antimicrobial resistance	AMR
Multidrug resistance	MDR
Minimum inhibitory concentration	MIC
National Animal Health Monitoring System	NAHMS
National Antimicrobial Resistance Monitoring System	NARMS

INTRODUCTION

Diseases caused by non-typhoidal *Salmonella enterica* have a major impact on human health. Globally, an estimated 93.8 million illnesses and 155,000 deaths are caused by non-typhoidal *Salmonella* annually (Majowicz et al. 2010). Within the United States, FoodNet actively collects data in 10 different states on laboratory-confirmed infections of seven different bacteria and two parasites (Voetsch et al. 2004). This system identified 41,000 laboratory-confirmed cases of salmonellosis in people, resulting in an incidence rate of 17.6 illnesses/100,000 persons, and a much higher incidence rate in children of 69.6/100,000 (CDC 2011a). *Salmonella* was associated with the greatest number of illnesses, deaths, and hospitalizations compared to other foodborne pathogens (CDC 2011c). The true incidence rate is likely much higher because of underreporting of cases. Estimates for the true disease burden of non-typhoidal salmonellosis in the U.S. range from 1 to 1.4 million illnesses per year, 378 deaths and over 19,000 hospitalizations (Mead et al. 1999; Voetsch et al. 2004; Scallan 2011).

The implementation of the control procedures and interventions at the harvest stage of the food chain has been associated with in declines in the incidence of most foodborne pathogens, including Listeria and E. coli O157:H7 (CDC 2011c; Tappero et al. 1993). The human health impact of salmonellosis in the United States, however, has increased over time. The incidence of salmonellosis was significantly higher in 2010 relative to 2006-2008 (CDC 2011c). Relative to the period of 1996-1998, the incidence in 2010 was not significantly different (CDC 2011c). The "Healthy people 2010" target (6.28/100,000) for reducing the incidence of salmonellosis was not met, and a new target of 11.4 cases/100,000 has been set for 2020 (CDC 2012b). A recent report

from the CDC states "*Salmonella* infection should be targeted because it has not declined significantly in more than a decade" (CDC 2011c).

Antimicrobial resistance (AMR) in *Salmonella* reduces treatment options for clinicians and veterinarians, and increases the morbidity and mortality of salmonellosis in humans (Maragakis et al. 2008; Varma et al. 2005). Patients infected with strains resistant to at least one antimicrobial are more likely to have septicemia (Varma et al. 2005a), and have significantly higher hospital costs (Maragakis et al. 2008). In human outbreaks of salmonellosis, 22% and 8% of patients infected with AMR and susceptible strains, respectively, necessitated hospitalization (Varma et al. 2005a). Among AMR *Salmonella* causing infections in people, resistance to tetracycline and sulfisoxazole is most common, but multidrug resistance (MDR) is present in 15.3% of *Salmonella* isolates (CDC 2012c).

Table 1- Most common serotypes recovered from laboratory confirmed cases of *Salmonella* in humans.

Serotype	**Number of Cases**	**Percent of total *Salmonella***	**Incidence per 100,000 persons**
Enteritidis	1,233	17.6	2.6
Typhimuirum	1,029	14.7	2.2
Newport	775	11	1.7
Javiana	550	7.8	1.2
Heidelberg	232	3.3	0.5
Montevideo	216	3.1	0.5
I:4,[5],12:i:-	210	3	0.4
Muenchen	172	2.4	0.4
Saintpaul	158	2.2	0.3

Adapted from - CDC. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet Surveillance Report for 2009 (Final Report). Atlanta, Georgia: U.S. Department of Health and Human Services, CDC. 2011.

Livestock farms are substantial reservoirs of *Salmonella* that cause infections in people. Specifically, dairy farms harbor zoonotic *Salmonella* subtypes that are important to public health. Although over 2,500 serotypes of *Salmonella* have been identified (Brenner et al. 2000), nearly half of the laboratory confirmed infections in humans were caused by four different serotypes (), including Enteriditis, Typhimurium, Newport, and Javiana (CDC 2011a). There is substantial overlap in the serotypes recovered from cattle, and those that cause illness in humans (**Table 2**) (Brichta-Harhay et al. 2011). Common serotypes from humans include those that have been recovered from cattle, including Typhimurium, Newport, Braenderup, Oranienburg, Thompson, and Mbandaka (USDA 2011b). *Salmonella* with identical AMR phenotypes have been recovered from cattle and humans (Wedel et al. 2005; Davis et al. 1999; Berge et al. 2004; Gupta et al. 2003; Zhao et al. 2003a). Additionally, *Salmonella* with indistinguishable genotypes have been recovered from cattle and humans (Zhao et al. 2003b; Soyer et al. 2010; Alcaine et al. 2006; Hoelzer et al. 2010).

Table 2 - Frequency of serotypes recovered from healthy cattle, clinically ill cattle, and clinically ill humans

Healthy Cattle		Bovine Clinical Cases		Human Clinical Cases	
Serotype	**% (No.)**	**Serotype**	**% (No.)**	**Serotype**	**% (No.)**
Cerro	28 (157)	Newport	12 (436)	Typhimurium	17 (6872)
Kentucky	23 (130)	Typhimurium Orion var.	11 (425)	Enteritidis	17 (6740)
Montevideo	12 (66)	15+,34+	10 (365)	Newport	8 (3373)
Mbandaka	8 (47)	Dublin	9 (335)	Heidelberg	4 (1495)
Meleagridis	7 (40)	Montevideo	8 (293)	Javiana	4 (1433)
Derby	5 (27)	Agona	6 (239)	I4,[5],12:i-	3 (1200)
Muenster	3 (18)	Anatum	6 (210)	Montevideo	3 (1061)
Anatum	3 (17)	Kentucky	4 (164)	Muenchen	2 (753)
Senftenberg	2 (13)	Muenster	4 (163)	Oranienburg	2 (719)
Newport	2 (11)	Cerro	4 (155)	Mississippi	1 (604)
Other	5 (30)	Other	26 (985)	Other	40 (16416)

Reprinted from USDA. 2011. *Salmonella, Listeria*, and *Campylobacter* on U.S. Dairy Operations, 1996–2007. USDA–APHIS–VS, CEAH. Fort Collins, CO

Important routes of transmission from cattle to humans include foodborne transmission and direct contact. Numerous large outbreaks due to direct contact have been documented, including outbreaks following contact with animals (Bender et al. 2004). Acquisition of AMR *Salmonella* infections through direct contact have also been attributed to cattle, including a ceftriaxone-resistant infection in a child (Fey et al. 2000). Nonetheless, transmission by direct contact has considerably less public health importance than the contamination of raw meat and poultry which are considered “the primary point of entry” for *Salmonella* into human populations (Sarwari et al. 2001b). Foodborne transmission represents approximately 95% of cases of illnesses caused by *Salmonella* (Mead et al. 1999; Scallan 2011). Beef and dairy products account for a substantial portion of *Salmonella* outbreaks where the vehicle of origin is known (Lynch et al. 2006). Outbreaks of *Salmonella* have been directly associated with the consumption

of beef and milk, particularly with the consumption of raw milk (Cody 1999; Tacket et al. 1985; Oliver et al. 2009; Mazurek et al. 2004). A massive outbreak of salmonellosis sickened over 16,000 people in the U.S, and was associated with pasteurized milk (Ryan et al. 1987). Large outbreaks of MDR *Salmonella* Typhimurium and Newport have also been associated with the consumption of beef (Dechet et al. 2006; Spika et al. 1987; CDC 2002). Although clearly an important source, it is not clear what proportion of the disease burden in humans can be attributed to cattle. Estimates for source attribution are inherently inaccurate, due the ability of *Salmonella* to disseminate to between and within animal populations. There is also substantial overlap in serotypes and subtypes between animal populations. The distribution of serotypes between slaughter isolates and human clinical isolates is different, suggesting that some of the serotypes of *Salmonella* shed by cattle pose less of a public health hazard (Sarwari et al. 2001b). Some of the bovine serotypes that are most frequently found on dairy farms or on carcasses are uncommon or rare causes of disease in humans (**Table 2**) (USDA 2012b; USDA 2011b). This discrepancy may be due to differences in virulence or differences in exposure.

Dairy cattle make an important contribution to the *Salmonella* disease burden in humans. Serotypes of *Salmonella* found in dairy cattle with high public health importance include Typhimurium and Newport. These strains of *Salmonella* emerged and were subsequently globally disseminated (Davis et al. 2002). Based on historical evidence, continued emergence of antimicrobial resistant and/or virulent subtypes can be expected. Additional epidemiologic and molecular research should be directed towards the entire population to understand the epidemiology, ecology and evolution associated with changes in the prevalence and *AMR* of *Salmonella* within livestock populations.

Problem Statement

There have been important changes in the prevalence and AMR of *Salmonella* in consecutive cross-sectional studies by the national animal health monitoring system (NAHMS). The proportion of samples positive for *Salmonella* from adult dairy cattle roughly doubled between 1996 and 2007 (USDA 2011). Concurrently, the AMR of *Salmonella* decreased between the most recent NAHMS studies in 20002 and 2007. These findings suggest important changes in the population of *Salmonella* on dairy farms over the past 10 years; however, data on long-term within farm changes in the population of genotypes that may explain associated changes in prevalence and AMR are not available. Changes in AMR may be due to gain or loss of AMR genes within the same genetic lineage. Alternatively, changes in the relative prevalence of different phylogenetic lineages may also lead to changes in prevalence estimates for AMR.

Overall research questions

The overall aim of this dissertation work is to determine the association of *Salmonella* population changes and changes in prevalence and AMR in Michigan dairy farms between 2000-2001 and 2009. Specifically, there are four research questions this research aims to address:

1) How has the prevalence of *Salmonella* on Michigan dairy farms changed over the past 10 years?
2) How has the AMR of *Salmonella* changed within-farms between 2000-2001 and 2009?

3) How has the population of genetic subtypes changed within farms, and what is the genetic relatedness of *Salmonella* recovered from the same farms between time periods?

4) Are the changes in population of *Salmonella* associated with changes in AMR?

Overall Hypothesis

Genotypic changes in the population of Salmonella on Michigan dairy farms are associated with changes in the antimicrobial susceptibility.

Objectives

1) Determine overall and within-farm changes in the prevalence of Salmonella between 2000-2001 and 2009

2) Determine the type and distribution of changes in AMR of Salmonella isolated from Michigan Dairy farms in 2000 and 2009

3) Use serotype identification, pulsed field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) to determine changes in the population and genetic relatedness of Salmonella from Michigan dairy farms in 2000 and 2009

4) Determine the association of identified genotypic population changes with changes in antimicrobial susceptibility.

CHAPTER ONE

A Review of the Epidemiology of *Salmonella* and Antimicrobial Resistance in *Salmonella* on Dairy Farms

Introduction

The objective of the following literature review is to interpret the epidemiological literature on *Salmonella* that sheds light on the overall changes in the prevalence and AMR of *Salmonella* on dairy farms. Literature regarding important characteristics of *Salmonella* strains, animal-level associations, and farm-level associations with the farms will be reviewed. Subsequently, literature discussing temporal changes in the prevalence and AMR of *Salmonella* will be reviewed.

Characteristics of *Salmonella*

General Characteristics and Taxonomy

Salmonella enterica is a flagellated, gram-negative, facultative anaerobic bacteria with a complex species, subspecies taxonomic structure (Brenner et al. 2000). Over 2,500 serotypes of *Salmonella* have been identified (Brenner et al. 2000). Historically, each serotype was considered a distinct species; however, definitive DNA hybridization studies showed only two distinct species of *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. Within *Salmonella enterica*, six subspecies were created: *Salmonella enterica* subspecies I – VI. The first subspecies, named *enterica*, has the greatest number of serotypes, and is most properly referred to as *Salmonella enterica* subsp. *enterica* (Brenner et al. 2000). For simplicity, the genus and serotype are used together, omitting the species and subspecies designation. For instance, *Salmonella enterica*

subsp. *enterica* serotype Typhimurium is commonly referred to as *Salmonella* Typhimurium (Brenner et al. 2000).

Within subspecies I (subsp. *enterica*), the Kauffman-White scheme is used to further classify organisms into serotypes according to the cell wall antigens (O-grouping) and flageller antigens (H grouping) (Kauffman 1975). Over 90% of *Salmonella* can be categorized as serogroup A, B, C1, C2, D, or E. Serogroups are further subdivided into serotypes using serologic identification of phase 1 and phase 2 flageller antigens, encoded by *fliB* and *fliC* genes (Iwen 1996). Alternating expression of each gene may require phase reversal to identify the serotype of the organism. Serotypes define important host-specificities or host-adapted strains (Sukhnanand et al. 2005). Serotyping provides a useful, "epidemiologically congruent" albeit, old method for grouping *Salmonella* (Liu et al. 2011). The distribution of the associated genes for pathogenicity, virulence, and AMR are different across different serotypes (Beutlich et al. 2011; Stevens et al. 2009). Additionally, the genetic mechanisms for diversification of a strain, including the relative influence of mutations and homologous recombination(Sangal et al. 2010; Lan et al. 2009) differ across serotypes.

Subtyping Methods for *Salmonella*

Serotyping as a method of discrimination for *Salmonella* has disadvantages, and deeper understanding of the epidemiology and ecology of *Salmonella* often requires distinctions within serotypes. For instance, MDR and susceptible phylogenetic lineages of *Salmonella* Newport are specific to bovine and poultry populations, respectively (Zhao et al. 2003b). Some phylogenies within serotypes don't have little in common except the serotyping antigens (Sangal et al. 2010). Serotypes may "confound" evolutionary histories through the later transfer of flagellar genes,

and because of these disadvantages MLST has been suggested as a replacement for serotyping (Achtman et al. 2012). Emerging applications of gene sequencing techniques and application of enzymatic and electrophoresis techniques have enhanced the knowledge of the diversity of *Salmonella enterica*. In general, the use of subtyping techniques has three primary goals. First, subtyping techniques can be used to provide epidemiologically relevant groupings of isolates. Techniques such as MLST often have lower discriminatory power that other subtyping techniques including PFGE (Kotetishvili et al. 2002), but are useful to provide important and interpretable categories of *Salmonella*. Second, techniques with higher power for differentiation, including PFGE and whole genome sequencing, are useful for measuring strain diversity or source attribution. Lastly, subtyping techniques can be used to provide phylogenetic inferences, and to study the evolutionary mechanisms that lead to the emergence of novel subtypes (Boxrud 2010). Categories of subtyping techniques include phenotypic methods, restriction enzyme digestion-based methods, PCR-based methods, and gene sequencing methods (Foley et al. 2007). Examples of phenotypic methods include phage typing, multilocus enzyme electrophoresis (MLEE), and AMR profiling. Phage typing relies on the selective ability of bacteriophages to infect certain strains of *Salmonella*. This technique has historically been useful to identify the globally disseminated *Salmonella* Typhimurium DT104 (Glynn et al. 1998); however, there are a limited number of available phages and some strains are untypeable by this method (Foley et al. 2007). Examples of restriction enzyme digestion-based methods include plasmid analysis, amplified fragment length polymorphisms (AFLP), and restriction fragment polymorphism analysis. Plasmid analysis involves isolation of plasmid DNA from the rest of the chromosome and separation of whole or restricted plasmids using electrophoresis. This technique is useful to identify the number and size of plasmids, but the rapid changes in plasmid profiles and the

responsiveness to selective pressure make this a useful subtyping technique only for studies of short duration (Foley et al. 2007). Restriction fragment length polymorphism analysis includes multiple techniques such as ribotyping and pulsed-field gel electrophoresis (PFGE).

PFGE is a highly discriminating technique that has been widely used to study the diversity of *Salmonella* recovered from humans and animals (Tenover et al. 1995; Barrett et al. 2006). It has historically been considered the "gold standard" of molecular techniques, owing to the laboratory reproducibility and the ability to produce a "fingerprint" of the entire genome (Foley et al. 2007). PFGE utilizes rare-cutting restriction enzymes (e.g. *XbaI*, *BlaN*, etc.) to cleave the chromosome into an appropriate number of large DNA fragments. The number of fragments is small enough to create an interpretable banding pattern on a single gel. The alternating polarity (pulsed-field) of the electrophoresis current enables the migration of DNA fragments that would otherwise be too large to migrate through the gel (Ribot et al. 2006).

Classic standards for the interpretation of PFGE patterns suggest that three-band differences between patterns should be interpreted as "closely related" strains, and six-band differences should be interpreted as "possibly related" (Tenover et al. 1995). A three-band difference between two strains could theoretically result from the single point mutation in a restriction site. These criteria for interpretation were updated by Barrett et al., 2006, who emphasized using only high quality gels and interpreting a relationship between two strains in light of epidemiologic information and the known diversity of the organism in question. The percent similarity of two banding patterns is typically expressed using similarity coefficients, where the number of bands in common is divided by the total number of bands between the two strains. Typically, matrices of the similarity estimates are calculated, and dendrograms are constructed using hierarchical agglomerative clustering techniques. Although these similarity

measures are often reported as genetic relatedness, more recent evidence suggests that PFGE using a single enzyme provides a poor estimate of genetic relatedness. Computer simulated genetic sequences and simulated chromosome restriction showed inadequate correlation between the calculated similarity and the actual genetic similarity using the entire genome (Singer et al. 2004). Pearson correlation coefficients of similarity estimates using different restriction enzymes were low (0.40-0.80) (Zheng et al. 2011). Bands of the same size do not always represent the same genetic material (Davis et al. 2003), and insertions or deletions that are smaller than 1-2% of the total chromosome size may not visibly alter the position of the band (Barrett et al. 2006).

Despite these disadvantages, PFGE provides good ability to discriminate between two strains of *Salmonella*. The technique has been successfully used for over twenty years as part of the PulseNet program to identify geographically widespread outbreaks of Salmonellosis (Swaminathan et al. 2001). PFGE utilizes the entire genome, banding patterns remain stable over time, and the technique is reproducible across multiple laboratories (Foley et al. 2007). Interpreted in light of serotype and epidemiological data, the similarity estimates nonetheless provide a rough estimate of the relatedness of two organisms in question. To interpret phylogenetic relationships, PFGE should be coupled with other techniques, such as MLST (Hoelzer et al. 2010). More recently, researchers have used up to six enzymes improve the genetic inferences for PFGE data (Zheng et al. 2011).

PCR amplification techniques and gene-sequencing techniques address many of the short-falls of other subtyping techniques, but have their own disadvantages. Examples of PCR-based methods include Rep-PCR (repetitive element PCR) and MLVA (multilocus, variable number of tandem repeat analysis). MLVA techniques utilize variations in the length of repeated sequence motifs (Boxrud 2010), providing unambiguous data and high discriminatory capacity,

particularly for clonal serotypes such as *Salmonella* Enteritidis and Typhimurium (Alcaine et al. 2006; Cho et al. 2007). However, it is not applicable towards a diverse population of *Salmonella*, and the selection of repeated motifs and associated primers must be optimized for each serotype (Boxrud 2010). Examples of gene sequencing subtyping techniques include whole-genome sequencing, single-locus sequence typing, multilocus sequence typing (MLST), and single nucleotide polymorphism (SNP). For *Salmonella*, SNP analysis may not be sufficiently discriminating, and SNP's must be identified through whole genome sequencing for several members of the species or serotype (Boxrud 2010).

MLST has become more commonplace for subtyping foodborne pathogens, particularly *Salmonella*. The gene sequence of multiple selected loci within the organism is determined and compared. These data are unambiguous and provide valid phylogenetic inferences. The amount of capacity for differentiation of strains is in part determined by the selection of the loci and the number of loci sequenced. Generally, housekeeping loci are used because they are present in all isolates of the species and are not subject to selective pressures that might result in rapid changes (Foley et al. 2006a). Early studies of the application of MLST towards *Salmonella* species used difference loci schemes and found different levels of discrimination. Studies using virulence or pathogenicity loci found the levels of discrimination to be similar to PFGE (Kotetishvili et al. 2002; Foley et al. 2006). Contrary to this, Fakhr et al. 2005 did not find any sequence variation after applying a four-gene MLST scheme towards a diverse collection of *Salmonella* Typhimurium. Other studies have found the discriminatory capacity to be less than PFGE (Sukhnanand et al. 2005; Torpdahl et al. 2005; Litrup et al. 2010). Despite this disadvantage, MLST is applicable towards a diverse collection of *Salmonella*, provides unambiguous data, and is useful to discern evolutionary histories.

Population structure

Salmonella has been called the "paradigm of a clonal species" by population geneticists, mainly because of the role of point mutations in the accumulation of genetic diversity (Wiesner et al. 2009). More recent gene sequencing studies, however, suggest that the role of recombination plays a larger role in the evolution of *Salmonella* than previously thought (Sangal et al. 2010; Didelot et al. 2011). These studies also show that the role of diversification differs by serotype (Sangal et al. 2010; Lan et al. 2009). Using gene sequencing data from seven housekeeping genes, the ratio of mutations to recombination differed for different serotypes. *Salmonella* Typhimurium and Enteritidis accumulated sequence diversity primarily through point mutations (Lan et al. 2009), while *Salmonella* Newport and Kentucky contained multiple distinct lineages that arose through homologous recombination within serotypes (Sukhnanand et al. 2005; Sangal et al. 2010). The well-studied MDR *Salmonella* Newport was specific to one of three lineages within Newport.

Virulence

Salmonella can cause asymptomatic colonization or clinical disease ranging from mild enteritis to severe life-threatening septicemia. The diversity and distribution of the virulence genes are important to understand the epidemiology of *Salmonella* dairy farms. *Salmonella* has several key mechanisms for establishing infections and causing disease. Survival at low pH enables passage through the acidic environment of the stomach into the intestines (Foley & Lynne 2008). Fimbrial attachment to a variety of host intestinal cells types is mediated through a Type III secretion system (Ibarra & Steele-Mortimer 2009), which is encoded on the *Salmonella* pathogenicity island II (SPI2) (Foley & Lynne 2008). SPI1 and SPI2 also encode for mechanisms for intracellular survival in macrophages (Steele-Mortimer 2008), within the

Salmonella containing vacuole (SCV). Systemic spread is most frequently associated with survival within dendritic cells (Foley & Lynne 2008). Differential expression of genes within the pathogenicity islands is often associated with changes in osmolarity, different nutrient levels, and acidification of the SCV (Foley & Lynne 2008). The self-transferrable *Salmonella* virulence plasmid is required for systemic disease (Rotger & Casadesús 1999), and encodes two genes, *spvB* and *spvC*, which are important for host cell cytotoxicity (Ibarra & Steele-Mortimer 2009).

Virulence is not a property of all strains of *Salmonella* (Gebreyes et al. 2009). The distribution of *Salmonella* serotypes in livestock differs from the distribution of serotypes causing disease in humans (Sarwari et al. 2001), suggesting that some serotypes may be better adapted for asymptomatic colonization than causing disease. Pathogenicity and virulence genes are distributed differently across different serotypes (Beutlich et al. 2011; Stevens et al. 2009). All European serotypes possessing *Salmonella* genomic island-1 (SGI-1) also possessed the virulence genes that are associated with *Salmonella* pathogenicity island-1(SPI-1) (Beutlich et al. 2011). Cluster analysis using the presence/absence of virulence genes encoded within the SPI1 showed that strains clustered according to serotype (Litrup et al. 2010). Although the SPI's are present in most serotypes, the distribution of the virulence plasmid is more specific. Genes associated with the virulence plasmid were located on Typhimurium, Enteritidis, and Dublin, but not Derby, Java, or Saintpaul (Litrup et al. 2010). The *spvA* gene, contained within the virulence plasmid, was found primarily within *Salmonella* that were isolated from clinically ill patients, rather than surveillance isolates (Gebreyes et al. 2009). Beutlich et al. 2011, showed that all Typhimurium isolates contained genes associated with the virulence plasmid, but plasmid-associated virulence genes were absent in other SGI-1 positive serotypes, including Newport, Kentucky, and Derby. Other research has shown variation in the presence or absence of virulence

genes within serotypes (Litrup et al. 2010). For an emerging strain of *Salmonella* Cerro on dairy farms, the *spvA* gene was absent (Cummings et al. 2010). Genes associated with the virulence plasmid were more frequently found in Typhimurium isolates from humans relative to Typhimurium isolates from animals (Wiesner et al. 2009). Strains that carry the virulence plasmid are also capable of switching between less virulent and hypervirulent states (Heithoff et al. 2012).

Antimicrobial resistance

The emergence of AMR strains of *Salmonella* has had an important impact on animal and human health. The term "antimicrobial resistance" can be used in different ways, including therapeutic failure, innate resistance, high MICs (minimum inhibitory concentrations) relative to the population distributions, and the presence of genetic resistance determinants. For the purposes of this literature review, however, AMR refers to the acquisition of phenotypic resistance in a previously susceptible bacterium (Alcaine et al. 2007).

AMR in *Salmonella* reduces treatment options for physicians and is associated with higher mortality, invasiveness, and higher hospital costs (Maragakis et al. 2008; Varma et al. 2005a). The emergence of resistance to 3rd generation cephalosporins in dairy cattle has particular relevance for human health. Ceftriaxone is the treatment of choice for invasive *Salmonella* infections in children, where fluoroquinolones are contraindicated. Resistance to other antimicrobials is clinically significant due to the ability of *Salmonella* to transfer AMR genes to other species, making *Salmonella* a reservoir of AMR genes for other pathogens. (Alcaine et al. 2007).

Various methods have been utilized for measurement of the phenotypic resistance to antimicrobials, including disk diffusion, broth dilution, and broth microdilution. The lowest concentration of antimicrobial that inhibits growth of the bacteria is deemed the MIC, and the MIC's are categorized as susceptible, intermediate, or resistant based on interpretive critieria (breakpoints). Resistance to antimicrobials is not randomly distributed among *Salmonella*. Rather, many MDR patterns are commonly seen. The most common multidrug resistance phenotype among MDR *Salmonella* is the ACSSuT[1] phenotype (CDC 2012c). This phenotype was first identified in the globally disseminated *Salmonella* Typhimurium DT104 (Threlfall 2000). The second most common phenotype is the MDR AmpC phenotype, most frequently associated with *Salmonella* Newport, which includes the ACSSuT phenotype, as well as resistance to amoxicillin clavulanic acid and ceftriaxone (CDC 2012c). In humans and cattle, the majority of MDR phenotypes are associated with serotypes Typhimurium and Newport (Brichta-Harhay et al. 2011; CDC 2012c). The resistance patterns, while frequently associated with a clonal strain, are not specific to that strain. Thirty-six percent of the isolates with the ACSSuT phenotype were not DT104, and 33% of the isolates with the AmpC phenotype were serotypes other than Newport (CDC 2012c). Surveillance isolates of *Salmonella* Montevideo and Reading with variations of the AmpC phenotype were recovered in the most recent NAHMS study.

At the cellular level, there are three general mechanisms that result in resistance to antimicrobials: changing the target within the cell, prevention of entry of the antimicrobial into the cell, decreasing the intracellular concentration via active drug efflux, and degradation/inactivation of the antimicrobial (Tenover 2006). *Salmonella* organisms may possess

[1] Ampicillin, chloramphenicol, streptomycin, sulfisoxazole, tetracycline

one or more genes that confer resistance to the same antimicrobial. Although over 35 tetracycline resistance genes have been recovered, 5 genes *Tet* genes are typically found in *Salmonella*. *TetG*, in particular, is typically associated with SGI1, while *tetA* and *tetB* have been associated with conjugative transposons (Michael et al. 2006). Of particular importance to human health is fluoroquinolone and cephalosporin resistance. Resistance to quinolones (e.g. naladixic acid) is mediated through the accumulation of targeted mutations with gyrA and gyrB, rather than the presence or absence of a specific gene (Alcaine et al. 2007). Cephalosporin resistance is notable because of the clinical significance in human medicine, the interesting epidemiological features of the antimicrobial resistant strains, and the concern about the use of ceftiofur (3^{rd} generation cephalosporin) on dairy farms. Genetic mechanisms that confer resistance to ceftiofur also confer resistance to ceftriaxone, the drug of choice for treating invasive *Salmonella* infections in children (Alcaine et al. 2007). Due to concerns about use of ceftiofur in livestock and the spread of ceftiofur resistance genes, the FDA issued an order prohibiting certain extralabel uses of the drug (FDA 2012a). Cephalosporin resistance is most frequently mediated by either AmpC cephalosporinases or extended spectrum β-lactamases (ESBL's), encoded by the CMY-2 and CTX-M genes, respectively (Geovana Brenner Michael et al. 2006). CTX-M enzymes are of particular concern because they are capable of hydrolyzing the β-lactam ring on 4^{th} generation cephalosporins. On continents other than North America, TEM and SHF β-latamase enzymes have been supplanted by the dissemination of CTX-M ESBL's (FDA 2012a; Frye et al. 2008). In the United States however, CMY-2 enzymes are the predominant enzymes associated with ceftriaxone resistance (Frye et al. 2008). Concerns exist that clonal and horizontal spread of genes encoding CTX-M enzymes will increase the prevalence of resistance to clinical important

cephalosporins in the United States. Indeed, CTX-M genes have recently been recovered from *E. coli* in a livestock market in Ohio (Wittum et al. 2010).

The locations of AMR genes within *Salmonella* are important for understanding the changes in AMR. Horizontal transfer of resistance can occur between species and between *Salmonella* serotypes (Alcaine et al. 2007). Transfer of CMY2 plasmids between *E. coli* and *Salmonella*, and between animal and human bacteria has been documented (Winokur et al. 2001). In *Salmonella*, AMR gene transfer primarily occurs through transfer of plasmids and class one integrons (Alcaine et al. 2007). Many genes, including the genes conferring the AmpC phenotype, are plasmid based (Chen et al. 2004). Location of AMR genes near transposons allows switching between chromosome and plasmid locations (Rychlik et al. 2006), while integron associated gene cassettes allow enzymatic incorporation of addition AMR genes (Rychlik et al. 2006). The integration of the functions of plasmids, integrons, and transposons results in a unique ability for AMR genes to pass between bacteria. Indeed, the epidemiology and ecology of AMR genes are somewhat unique from their host organisms (Wiesner et al. 2009). Integrons can be located within transposons, and transposons can be located within plasmids. In this way, an integron can capture new genetic material, the plasmid on which it resides can be conjugatively transferred to a new bacterium, and the plasmid transposed into the chromosome of its new host.

The presence of multiple genes on the same plasmid or integrating element results in co-selection and/or the simultaneous transfer of resistance to multiple antimicrobials. Integron-dependent AMR genes are a key component of the horizontal transfer of AMR in *Salmonella* (Alcaine et al. 2007; Rychlik et al. 2006; Hall et al. 1996). *Salmonella* genomic island 1 (SGI-1) is frequently detected within *Salmonella*, and most commonly contains genes that confer the

ACSSuT phenotype. Genes within SGI-1 can be excised and conjugatively transferred through the action of integrases (Doublet et al. 2005). SGI-1 was detected for the first time within *Salmonella* Typhimurium DT104, but was later found in other serotypes, including Agona, Newport, and Albany. Indistinguishable integrons were also found in genetically unrelated isolates in a global collection of *Salmonella* that included clonal isolates found on different continents (Krauland et al. 2009). Genetic variants of SGI-1 are associated with different serotypes (Beutlich et al. 2011), suggesting that diversification of SGI-1 may differ by serotype.

AMR in *Salmonella* is associated with virulence. AMR isolates are more likely to have virulence genes (Gebreyes et al. 2009), and isolates originating from clinically ill patients are more likely to be MDR (FDA 2012b). Resistance to quinolones was associated with the invasiveness of the infection caused by *Salmonella* (Helms et al. 2004). Plasmids carrying virulence or AMR genes are structurally related, and may be spread on the same plasmid (Fricke et al. 2009).

Environmental survival

Survival and replication within the environment has important implications for understanding the epidemiology on dairy farms. *Salmonella* has unique abilities to sustain environmental stressors, including pH changes, dessication, freezing, and low nutrient availabilities. Prior studies have shown survival in manure (You et al. 2006; Nicholson et al. 2005) for up to 26 and 47 weeks in manure and manure-amended soils. Concentrations of *Salmonella* initially increased following inoculation into manure lagoon effluent. The upper limit for the duration of persistence of *Salmonella* in manure effluent isn't known, because the study was terminated before the concentration of *Salmonella* had decreased (Toth et al. 2011).

Salmonella Newport survived longer in a manure lagoon than a compost pile of soil or grass (Toth et al. 2011). Other studies have shown longer survival in manure slurry than manure (Himathongkham et al. 1999) and no difference between survival within different types of manure compost (poultry or dairy) (Islam et al. 2004).

Animal-level Epidemiology of *Salmonella* on Dairy Farms

The unique attributes of *Salmonella* play a decidedly important role in its epidemiology. However, the outcome of infection (asymptomatic colonization, disease, and severity of disease) depends on host characteristics (Stevens et al. 2009). The objective for this section of the literature review is to highlight pertinent animal-level (host) epidemiological features of *Salmonella* in dairy cattle. Differing study designs and hypotheses often make direct comparisons difficult. Many studies that focus on shedding in healthy cattle (Fossler et al. 2004; Huston et al. 2002; Kabagambe et al. 2000; Blau et al. 2005; Ruzante et al. 2010) recover different populations of *Salmonella* than studies that focus on isolates from clinically ill animals (Warnick et al. 2003; Cummings et al. 2009b; Alcaine et al. 2006).

Prevalence of Shedding

Within farms, the proportion of healthy cattle shedding *Salmonella* can vary between 0 and 100% (Edrington et al. 2004). Averaged across farms, the proportion of adult dairy cattle shedding *Salmonella* was 5.4%, 7.1% and 13.7% in studies by the National Animal Health Monitoring System (NAHMS) in the years 1996, 2002, and 2007. Consistent with the NAHMS studies, a study on Midwestern dairy farms showed prevalence estimates of 4.9% and 6% (Fossler et al. 2005; Huston et al. 2002). In a four-state study of *Salmonella* shedding, Fossler (2005) recovered *Salmonella* from 4.9% of adult cattle, which was somewhat higher than the

proportion of calves that were shedding in the same study (3.8%). Meanwhile, 8.3% of calves were shedding *Salmonella* in a study conducted in the Western United States (A. C. B. Berge et al. 2006). Few studies of the incidence of clinical illness have been done. In one such study, however, the incidence of clinical illness was higher in calves (8.1/1000 animal-years) than in cows (1.8 cases/1000 animal years) (Cummings et al. 2009b).

Animal-level risk factors

Although healthy cows were more likely to be shedding *Salmonella* in previous research (Fossler et al. 2005; Huston et al. 2002), calves were more likely than cows to have a positive sample on farms that had previously had clinically ill animals diagnosed with serogroup B salmonellosis (Warnick et al. 2003). These discrepant results may have to do with the differing characteristics between clinical and surveillance isolates of *Salmonella*. Calves have been found to be more likely to shed *Salmonella* that are AMR (Wells et al. 2001). In a longitudinal study of *Salmonella* shedding by preweaned calves, the prevalence of shedding steadily decreased as calves got older; 18% of 1 day old calves were shedding , which decreased to 0% by weaning (Berge et al. 2006). In cows, identified risk factors have included multiparity and an early stage of lactation (Fitzgerald et al. 2003). Fossler et al., 2005, however, did not find an association with state of lactation (Fossler et al. 2005), and an association with the middle of lactation was found in other research (Huston et al. 2002). Cows that are slated for culling have been found to be more likely to be positive in one study (Wells et al. 2001), although in another study, shedding by cows slated for culling was numerically, but not statistically significantly higher (Fossler et al. 2005). In three cross-sectional studies from NAHMS, 18% (121/668), 0% (0/17), and 12.6% (17/135) of cull cows were positive for *Salmonella*. The high prevalence of *Salmonella* in cull dairy cattle has been suggested to be due to transportation stress (Beach et al. 2002) or changes

in feeding behavior (Wells et al. 2001). Indeed, *Salmonella* is capable of responding to host-secreted catecholamines with increased virulence and growth activation (Stevens et al. 2009). Other research, by contrast, has shown that the proportion of rectal fecal samples positive for *Salmonella* did not change between the feedlot and lairage. Rather, the proportion of hides positive increased, suggesting no change in the proportion of animals shedding, but a higher level of contamination as a result of crowding (Beach et al. 2002; Barham et al. 2002). Fecal samples from cows classified as sick (for any reason), and environmental samples from sick pens were more likely to be positive for *Salmonella* than samples from healthy cows (Fossler et al. 2005). Similarly, the presence of diarrhea at the time of sampling and recent antimicrobial treatment were both significantly associated with the recovery of *Salmonella* (Warnick et al. 2003). Decreases in immune function associated with illness, or changes in feeding behavior may explain the association of *Salmonella* shedding and illness.

The association of *Salmonella* shedding with recent antimicrobial treatment has been a consistent finding in human and veterinary epidemiological studies. In separate case-control studies of outbreaks of *Salmonella* Typhimurium and Newport in people, study subjects that had taken an antimicrobial in the month prior to the outbreak had higher odds of illness (Ryan et al. 1987; Spika et al. 1987). Human illnesses during an outbreak of pansusceptible serotype Havana were associated with prior antimicrobial treatment (Pavia et al. 1990). A national-level case-control study of sporadic cases of MDR *Salmonella* Newport in humans found cases were more likely to have taken an antimicrobial in the 28 days prior to illness (Varma et al. 2006). In the veterinary literature, recent antimicrobial treatment was associated with salmonellosis in horses hospitalized at a veterinary teaching hospital during an outbreak. Serogroup B *Salmonella* (includes Typhimurium) were more likely to be recovered from heifers and cows that had

received antimicrobial treatment in the last 1-2 months (Warnick et al. 2003). Prophylactic treatment of calves with antimicrobials on the first day of life was associated with shedding *Salmonella* at any point in the preweaning period (Berge et al. 2006). The author noted, however, that this practice may have been put into place because of previous outbreaks of salmonellosis. Although a consistent finding across epidemiologic studies, the cause/effect relationship of antimicrobial therapy has not been clearly established (Warnick et al. 2003). Antimicrobial therapy disrupts the intestinal flora and decreases the diversity of the microbiome. This disruption of the microbial architecture may allow a new colonization of the intestines with *Salmonella*, or facilitate the proliferation of small populations of *Salmonella* already present in the gut. Alternatively, previous antimicrobial therapy may simply be an indicator of ongoing immune suppression or another disorder. However, immunosuppressive drugs, antacid use, or gastric surgery were not associated with infections with *Salmonella* Typhimurium (Ryan et al. 1987). In a human epidemiologic study, the association with antimicrobial use remained despite controlling for illness or immune suppression (Pavia et al. 1990). Longitudinal studies that examine the association between changes in the microbiome and *Salmonella* colonization will be useful to clarify the cause and effect relationship between antimicrobial therapy and shedding.

There are broad gaps in the knowledge of animal risk factors, and host associations with *Salmonella*. For instance, the host and environmental characteristics associated with persistence within animals or the environment is largely unknown. Given the diverse characteristics of *Salmonella* strains, there are likely important differences in the epidemiology across serotypes, serogroups or patterns of antimicrobial susceptibility. Variability in the quantity of organisms shed by *Salmonella* positive animals, and the external influences on that variability need to be further explored. *Salmonella* infections may result in severe systemic disease, or asymptomatic

colonization, yet the host-level characteristics that result in different infection patterns need to be further elucidated.

Dairy Farm-Level Epidemiology of *Salmonella*

Animal and host factors are important to understanding the epidemiology of *Salmonella*. However, in an agricultural setting, animals are house in groups, and management changes or other influences are often applied at the level of the herd. Therefore, it is useful to study the herd-level epidemiology of *Salmonella* on cattle farms, and the influence of management practices on *Salmonella* shedding and AMR. In cross-sectional studies of *Salmonella* shedding on dairy farms the proportion of herds positive for *Salmonella* has ranged from 21% to 40% (Kabagambe et al. 2000; Huston et al. 2002; Habing et al. 2012; Blau et al. 2005; USDA 2011b; T R Callaway et al. 2005). However, in a longitudinal study with samples taken every two months over a year, at least one *Salmonella* isolate was recovered from 90% of herds (Fossler et al. 2004). The herd-level prevalence is lower in calf ranches (5.8%) (Berge et al. 2006), but the incidence of clinical illness was higher in calves (8.1/1000 animal-years) than in cows (1.8 cases/1000 animal years). The herd-level incidence of clinical illness was 8.6 positive herds/100 herd-years (Cummings et al. 2009b).

Diversity

Diversity indices, including Simpson's index of diversity, are useful to understand the types, number, and the evenness of distribution of *Salmonella* on dairy farms (Hunter & Gaston 1988). Dairy farms that are positive for *Salmonella* are typically infected by a single predominant serotype, which is usually composed of a predominant pulsotype (Soyer et al. 2010). Estimates of diversity for *Salmonella* on dairy farms have been variable across studies,

and depend on the study design and methods for differentiation of isolates. Simpson's index of diversity of serotypes of surveillance isolates collected from cull dairy cattle from many herds ranged from 0.53 to 0.90, depending on the region and season (Galland et al. 2001). Some dairy farms harbor a broad range of serotypes. On two consecutive visits to a single dairy farm, 10 and 14 different serotypes representing 26 and 27 different genotypes were recovered, respectively. The diversity of serotypes and PFGE genotypes was not different between dry and lactating cows on a single dairy (Hume et al. 2004). The estimate of diversity using PFGE (0.991) was slightly higher than the estimate using MLST or serotyping alone (0.920 and 0.913, respectively) (Soyer et al. 2010). Another study in the Southwestern United States reported the Simpson's index of diversity for serotypes to be 0.811 (Callaway et al. 2005). In contrast to the diversity values within herds, MDR strains of *Salmonella* recovered from cull cattle at slaughter plants had diversity values of 0.1 – 0.5 (Brichta-Harhay et al. 2011).

Acquisition

External sources of *Salmonella* infections on farms may be from animal or feed imports, wildlife, or human traffic. Animal introductions are undoubtedly an important source of new introductions of *Salmonella* onto the farm, particularly for strains that cause clinical disease. Transmission of *Salmonella* from heifer feedlots back to the dairy have been documented (Edrington et al. 2008). Among 56 Western dairy herds, MDR strains were introduced onto the farm at a rate of 0.9/herd-year (Adhikari et al. 2009a). Off-site raising of heifers was significantly associated with the introduction of MDR strains, but not the number of purchased cattle (Adhikari et al. 2009b). This finding is consistent with research that shows young stock frequently harbor MDR *Salmonella* (Berge et al. 2006)

The role of feed in the transmission of *Salmonella* to cattle farms has been examined. The prevalence of *Salmonella* in feed samples from feed mills is very low (Davis 2003); however, rare accidental contamination at the feed mill has resulted in large and widely distributed outbreaks of serotypes Mbandaka, Menhaden, and Infantis (Jones et al. 1982; Anderson et al. 1997; Lindqvist et al. 1999). On the farm, recovery of *Salmonella* from feed piles, or feed storage units is more frequent. Approximately 0.2% , 42%, and 5.3% of feed on the farm was contaminated with *Salmonella* in two studies done on dairy farms and one in beef feedlots, respectively (Davis 2003; Dargatz et al. 2005; Kidd et al. 2002). However, the majority of the contamination was confined to a small number of piles. In an Oregon study of 32 dairy farms, 42% of the feed piles were positive for *Salmonella* (Kidd et al. 2002). Pulsotypes recovered from cattle on the farm were indistinguishable from those in the feed, demonstrating that strains were likely circulating between cattle and the feed (Davis 2003). Given the low prevalence at feed mills, and the substantially higher prevalence on farms, the presence of *Salmonella* in feed is likely a result of contamination of the feed on the farm, rather than introductions through feed mills.

Wildlife introductions of *Salmonella* may be an underestimated source of *Salmonella*. Contamination of haylage by birds was associated with shedding of *Salmonella* Anatum (Glickman et al. 1981). The number of starlings was associated with the prevalence of E.coli O157:H7 on dairy farms (Cernicchiaro et al. 2012). Although the prevalence of *Salmonella* among European starlings was low (Gaukler et al. 2009), other research documented a reduction in environmental contamination with *Salmonella* following starling-control programs (Carlson et al. 2011).

Persistence

Following introduction onto a farm, *Salmonella* must be able to establish itself in the microbial niche of the dairy farm environment and/or the gut of dairy cattle. In addition to acquisition of novel strains, the duration of infections and the persistence of those strains in the environment have a large influence on the dynamics of farm infections and within-farm prevalence estimates. *Salmonella* has a unique ability to persist within animals for long periods of time, and a carrier state has been described for *Salmonella* Dublin (McDonough et al. 1999). Other serotypes may cause long-term infections, however, and the shedding of *Salmonella* Newport by a single animal was documented for up to 190 days (Cobbold, D. Rice, et al. 2006).

At the farm level, long-term persistence is likely due to combinations of carrier animals, persistence in the environment, and temporary chain infections (Cobbold et al. 2006). Long-term persistence of *Salmonella* on farms has been repeatedly noted in the literature (Gay & Hunsaker 1993; Giles et al. 1989; Warnick et al. 2001; Cobbold et al. 2006). Strains causing an illness on dairy farms were recovered for up to eight months following an initial disease incident (Warnick et al. 2003). In calf barns, distinct strains of Typhimurium on 5 different calf raising units were recovered from 4 months to 2 years following the initial outbreak (McLaren & Wray 1991). Although *Salmonella* Newport persisted on a farm for greater than 6 months, only a single animal excreted the organism for the duration of the study, suggesting persistence on this farm was a combination of environmental persistence and chain infections, rather than extended excretions from a large number of animals (Cobbold et al., 2006).

The maximum and median observed duration of shedding of *Salmonella* following clinical disease has been perhaps best described by Cummings (2009a). Although there were large numerical differences in the duration of shedding within individual animals, the duration of

shedding did not differ across age groups or serotypes (Cummings et al. 2009). With a median duration of shedding of 50 days, and a maximum duration of over a year, the carrier state for *Salmonella* is a phenomenon that is not confined to the host-adapted serotype of *Salmonella* Dublin. Chronic shedding may be a result of the convalescent period after clinical disease, passive carriage as a result of acquisition from a contaminated environment, or shedding due to persistent infections in tissues (Stevens et al. 2009). Persistent infections in lymph nodes is likely play an important role, as approximately 1.6% of bovine lymph nodes were positive for *Salmonella* at slaughter. The prevalence of *Salmonella* in lymph nodes was higher in cull cattle compared to fed cattle, and the infecting strains included MDR Typhimurium and Newport (Arthur et al. 2008). Possible diversification of *Salmonella* strains and acquisition of AMR determinants during persistence within a dairy farm was also recently noted (Hoelzer et al. 2010).

The majority of the literature on persistence of *Salmonella* on dairy farms addresses the duration following clinical illnesses, primarily with serotypes of Typhimurium and Newport. None of the literature addresses the serotypes that are frequently asymptomatically shed in the feces of dairy cattle. Additional research on the host, strain, and farm characteristics that are conducive to persistence of *Salmonella* within farms will be important for complete understanding of the epidemiology of *Salmonella* on dairy farms.

Farm Level Risk Factors

Salmonella positive herds do not appear to be randomly distributed. Rather, the size of the herd and other management practices have consistently been associated with the herd prevalence. Additionally, a large burden of *Salmonella* shedding was attributable to a small

number of herds (Fossler et al. 2004). In all three NAHMS studies in 1996, 2002, and 2007, 10% of the operations accounted for over 75% of the positive samples. On-farm management practices or herd characteristics may influence the intestinal or dairy farm environment to provide conducive conditions for *Salmonella*. Epidemiological risk factors for the recovery of *Salmonella* from dairy farms can be divided into (non-mutually exclusive) categories of those that increase the likelihood of introduction (e.g. biosecurity practices), and those that may be conducive to persistence (Warnick et al. 2003). Risk factors may also be divided in the non-modifiable herd characteristics, such as region and herd size, and modifiable management practices that may represent future interventions.

For non-modifiable characteristics, there has been a consistent association with herd size. Larger dairy farms have been associated with a higher prevalence in adult cattle, (Habing et al. 2012; Ruzante et al. 2010; Kabagambe et al. 2000; Blau et al. 2005; Warnick et al. 2001), more frequent contamination of bulk tank milk (Ruzante et al. 2010), higher incidence of clinical salmonellosis (Cummings et al. 2009b), more frequent shedding in calves (Losinger et al. 1995), and a higher rate of introduction of MDR strains (Adhikari et al. 2009a). A portion of the association with herd size may be due to the use of targeted sampling, and the more frequent availability of animals most likely to be shedding *Salmonella*, including fresh cows, sick cows, and calves (Warnick et al. 2001). Notably, a large, 2-year longitudinal study of *Salmonella* shedding in the Midwest did not find a significant association with herd size while controlling for other management practices (Fossler et al. 2005a). The association with herd size may also be due to inherent differences in the management practices and biosecurity between small and large dairy farms. The positive association with herd size has been found in other livestock populations (Gardner et al. 2007), and for other bacterial species on livestock farms (USDA

2011a), suggesting that there are inherent differences in transmission dynamics between large and small populations of agricultural animals (Gardner et al. 2007). Higher *Salmonella* prevalence has been associated with the Southern region (Kabagambe et al. 2000; Wells et al. 2001; Blau et al. 2005), Western region (Blau et al. 2005), and Eastern region of the U.S. (Habing et al. 2012). The herd-level prevalence and contamination of hides has been associated with warmer months (Wells et al. 2001; Fossler et al. 2004; Brichta-Harhay et al. 2011), while in other studies shedding was higher in non-summer months (Losinger et al. 1995; Kunze et al. 2008). Production parameters, including individual milk, protein, and fat production were not associated with *Salmonella* shedding on dairy farms (Huston et al. 2002; Fossler et al. 2005).

Management practices that are causes of *Salmonella* shedding represent potential preharvest interventions. Previous findings on the association of *Salmonella* shedding with farm-level management practices are presented in **Table 3** and **Table 4**. Research into this area, however, has been inconsistent and sometimes contradictory. For instance, ionophores have either been positively or negatively associated with the *Salmonella* status of a herd, depending on the study (Habing et al. 2012; Ruzante et al. 2010; Fossler et al. 2005a). Consistently, however, the use of liquid manure has been associated with *Salmonella* shedding. Using a broadcast or solid spreader has been negatively associated with *Salmonella* shedding in multiple studies (Fossler et al. 2005a; Habing et al. 2012; Ruzante et al. 2010). These results are consistent with experimental work that has shown longer persistence of *Salmonella* in manure slurry relative to static manure piles (Nicholson et al. 2005; You et al. 2006; Toth et al. 2011). Manure in a liquid form may also be dispersed more broadly, making ingestion and colonization more likely (Fossler et al. 2005a). The consistent association across experimental and observational research

strengthens the potential causal association between manure management practices and *Salmonella* prevalence on dairy farms.

There is less research on the shedding of *Salmonella* in calves, possible because dairy calves, unlike adult cattle, do not routinely enter the food supply, and are supposed to pose less of a public health threat. However, it would be expected that *Salmonella* in the calf population would spill over into the adult cows, or contaminate adjacent agricultural fields. Nonetheless, the use of medicated milk replacer has been consistently associated with a reduction in the shedding of *Salmonella* (Fossler et al. 2005b; Berge et al. 2006; Losinger et al. 1995). This effect, however, may only be relevant for pansusceptible (and typically less virulent) strains of *Salmonella*.

Table 3- Farm-level management Practices associated with *Salmonella* shedding in dairy cows

Isolate type	Management Risk Factors	Manuscript
Surveillance	-Flush-water system -Feeding Brewer's Products	Kabagambe et al., 2000
Clinical cases	-Signs of rodents -Wild Geese contact with cattle or feed -Poultry manure spread on bordering property	Warnick et al., 2001
Surveillance	-Use of free stalls -Use of straw bedding	Huston et al., 2002
Surveillance	-No management practices significantly associated	Peek et al., 2004
Surveillance	-Lack of tie stalls for housing adult cattle -Not storing feed in an enclosed building -Disposal of manure in liquid form -Not using monensin in weaned calf or bred heifer diets -Eating or grazing roughage from fields where manure was applied	Fossler et al., 2005
Clinical	-No management practices significantly associated	Cummings et al., 2009b
Bulk Tank milk and Environment	-Not using a broadcast manure spreader -Use of bovine somatotropin -Use of anionic salts	Ruzante et al., 2010
Surveillance	-Sprinklers or misters for heat abatement -Feeding anionic salts to cows -Feeding ionophores to cows -Lack of use of broadcast/solid spreader	Habing et al., 2012

Table 4 - Farm-level management practices associated *Salmonella* shedding in dairy calves

Isolate type	Management Risk Factors	Manuscript
Surveillance	-Lack of routine feeding of medicated milk replacer -Not feeding hay from 24h to weaning -Being born in an individual area in a building	Losinger et al., 1995
Surveillance	-Lack of routine feeding of medicated milk replacer -Use of the maternity housing as a sick pen -Cow-level prevalence by visit	Fossler et al., 2005
Surveillance	-Open herds -Lack of feeding antimicrobials in the milk replacer -Prophylactic antimicrobials at the first day of age	Berge et al., 2006

Contamination of retail beef

Contamination of retail beef with *Salmonella* is low. Although the proportion of cattle hides positive for *Salmonella* just prior to slaughter has shown to be high (89%), the prevalence decreases through the slaughter process to 50.2% pre-evisceration and 0.8% in the chiller (Brichta-Harhay et al. 2011). The prevalence of *Salmonella* contamination in retail beef samples has been shown to be less than 2% (C. Zhao et al. 2001; LeJeune & Christie 2004; Mollenkopf et al. 2011), and routine surveillance by the USDA has shown that less than 2% of retail beef samples have been positive for *Salmonella* for each year between 2002 and 2010 (FDA 2012b). While this low prevalence may still have a large public health impact, it is small relative to *Salmonella* contamination in retail poultry products, where greater than 40% of chicken breasts and turkey samples were positive for *Salmonella* for each year between 2002 and 2010 (FDA 2012b). A Danish mathematical model estimated that eggs accounted for 38% of the domestic Danish cases of salmonellosis, while beef only accounted for 0.9% (Hald et al. 2007). A recent outbreak of MDR *Salmonella* Typhimurium was associated with ground beef, but many other

recent (2011-2012) multistate outbreaks of *Salmonella* have other sources, including small turtles, dry dog food, frozen raw yellow-fin tuna, feeder rodents, chicks and ducklings from a mail-order hatchery, and salami products made with contaminated imported black peppers (CDC 2012a). National-level epidemiologic studies of sporadic infections have identified previous antimicrobial therapy (Spika et al. 1987; Ryan et al. 1987; Pavia et al. 1990), consumption of ground beef, undercooked eggs, and contact with reptiles (Varma et al., 2006) as risk factors for Salmonellosis.

Epidemiology of Antimicrobial Resistance in *Salmonella* from Dairy Farms

Antimicrobial use on dairy farms has been postulated to cause increased resistance in human pathogens, and lead to the emergence of novel subtypes of *Salmonella*. Prevalence estimates for AMR in *Salmonella* shed by dairy cattle vary based on the study design and study population. Most commonly, studies either use surveillance isolates or collections of clinical isolates. Surveillance studies of AMR in livestock populations are designed to provide precise estimates that reflect the population present on livestock operations (USDA 2011b). Studies that use collections of *Salmonella* recovered from diagnostic specimens find different populations and higher frequencies of AMR, but do not accurately reflect the population present on the farm. Serotypes of *Salmonella* that are commonly isolated from cases of clinical disease include Newport, Typhimurium, and Dublin (Hoelzer et al. 2010). The most common serotypes recovered from surveillance studies of *Salmonella* on dairy farms include Montevideo, Muenster, and Anatum (USDA 2011b). The different distributions suggest that *Salmonella* that are asymptomatically shed are less likely to cause disease in humans. Regardless, interpretation of temporal trends in prevalence requires knowledge of the sampling methodology.

The prevalence of AMR in *Salmonella* from dairy cattle has typically been low, but variable between regions of the United States. Ray et al. 2007 demonstrated that the majority of isolates (1223/1506) from Midwestern and Northeastern dairy farms were susceptible to all tested antimicrobials, but 24% herds harbored at least one resistant isolate. Peek et al. 2004 found only pansusceptible *Salmonella* on herds that did not have a history of clinical disease. In the Southwest, 22% of isolates from six large dairy herds were resistant to at least one antimicrobial. Prior studies by NAHMS are designed to provide national-level prevalence estimates. In studies done in 1996, 2002, and 2007, 92.3%, 82,3%, and 96.6% of isolates were susceptible to all of the tested antimicrobials, respectively (USDA 2011b). NAHMS studies, however, only sample adult cows, and may underestimate the prevalence of resistance on dairy farms. Compared to the low prevalence of MDR in *Salmonella* shed by adult cows (3%-5%), 25% and 33% of *Salmonella* isolates from calves in the Midwest and California, respectively, were multidrug resistant (Berge et al., 2006; Ray et al., 2007).

AMR among *Salmonella* recovered from clinically ill animals is much more frequent. Among cows and calves diagnosed with salmonellosis on dairy farms, between 65% and 77% of the isolates were multidrug resistant (Ray et al. 2007; Cummings et al. 2009b). MDR in *Salmonella* was numerically higher among sick cows relative to healthy cows (Ray et al. 2007), possibly due to the co-location of AMR genes and virulence genes (Gebreyes et al. 2009). Prior to 2006, the national antimicrobial resistance monitoring system (NARMS) provided information on the serotypes and AMR of *Salmonella* recovered from diagnostic specimens from dairy cattle. Notably, 43% of the isolates were resistant to ceftiofur and ceftriaxone in 2006. Increases in resistance to these antimicrobials coincided with increases in the prevalence of *Salmonella* Newport.

Associations with Antimicrobial Resistance in *Salmonella* from Dairy Farms

Regional differences in the prevalence and type of antimicrobial resistance have been noted in multiple studies. The prevalence of MDR *Salmonella* recovered from the hides of cull cattle in slaughter plants varied according to the region (Brichta-Harhay et al. 2011). There was a higher prevalence of MDR *Salmonella* Typhimurium in two regions compared to two other regions in cull cattle hides sampled at slaughter. *Salmonella* Typhimurium DT104, however, was widely distributed across multiple regions (Brichta-Harhay et al. 2011). In a separate study using isolates from veterinary and human diagnostic laboratories, *Salmonella* from the Northwest were more likely to be MDR than *Salmonella* from the Northeast (Hoelzer et al. 2010). Similar regional associations have been noted for *E.coli*: isolates from dairy farms in California were more likely to be MDR than isolates from Oregon or Washington (Berge et al. 2010).

AMR in *Salmonella* has been associated with large dairy herds relative to smaller herds (Ray et al. 2006; Cummings et al. 2009b; Adhikari et al. 2009b). However, few management practices (excluding antimicrobial use) have been associated with AMR in *Salmonella*. Feeding or other management practices were not significantly associated with shedding of MDR *Salmonella* in preweaned calves (Berge et al. 2006)**.** Following an intervention (withdrawal of milk replacer), the proportion of *Salmonella* with a MDR phenotype was less in intervention herds than control herds, but trends in resistance following the intervention were not clear (Kaneene et al. 2009). There were no significant changes in the tetracycline susceptibility of *Salmonella* following the withdrawal of antimicrobials from the milk replacer (Kaneene et al. 2008). Another study addressing potential risk factors for AMR in *Salmonella* found associations with use of dried manure solids; however, the mechanism for this is not clear, and may represent a chance finding (Habing et al. 2012)**.** Using isolates of from clinically ill dairy cattle diagnosed

by private veterinarians on New York dairy herds, MDR was associated with herd size but not associated with other management practices (Cummings et al. 2009b).

Antimicrobial use in the dairy industry may lead to increases in AMR in *Salmonella*. The dairy industry has been scrutinized for the use of antimicrobials, particularly 3rd generation cephalosporins. The frequency of resistance to 3rd generation cephalosporins is particularly high among dairy cattle diagnostic samples (USDA 2006; Daniels et al. 2009). Resistance has been found to be higher among clinical isolates from cattle relative to clinical isolates from humans. Approximately 85% and 49% of bovine and human *Salmonella* clinical isolates, respectively, were MDR in one study (Hoelzer et al. 2010). Likewise, 44% and 12% of bovine and human *Salmonella* Typhimurium within a single PFGE clade were resistant to ceftazidime (Adhikari et al. 2010). *Salmonella* from dairy farms have been found to have higher levels of resistance than *Salmonella* from beef farms. *Salmonella* Dublin isolates from dairy origin were more likely to be AMR than Dublin isolates from beef origin (Davis, Hancock, et al. 2007), and beef feedlots in a dairy intense region had higher levels of MDR *E. coli* isolates than beef feedlots that were remote from dairy farms (Berge et al. 2010).

Results of previous research on the association between antimicrobial use and AMR in *Salmonella* on dairy farms are mixed. In one study, there was no association between ceftiofur use and AMR in *Salmonella* or *E. coli* (Daniels et al. 2009). In another study, however, there was a positive association of ceftiofur use with the MIC level of *E. coli* on Ohio dairy farms (Tragesser et al. 2006). Ray (2007) found somewhat higher levels of AMR on conventional herds relative to organic herds, but the effect was present for only a few antimicrobials, and one antimicrobial (naladixic acid) had higher levels of resistance on organic farms relative to conventional dairy farms. The lack of clear association with antimicrobial use and AMR in

Salmonella may not be surprising. As discussed later in this literature review, changes in AMR often occur through clonal dissemination. Due to the epidemiology of these clones, changes in AMR may not occur rapidly enough to discern differences between farms with different levels of antimicrobial use (Davis et al. 2002).

Changes in the Prevalence and Antimicrobial Resistance of *Salmonella*

Human Trends in Incidence and Antimicrobial Resistance

There have been important changes in the population of serotypes and AMR of *Salmonella* that cause infections in humans. The incidence of Salmonellosis in 2010 was not different than the incidence between 1996-1998 (CDC 2011c); however, the relative prevalence of serotypes causing the infections has changed. For instance, the incidence of infections was 53% lower for *Salmonella* Typhimurium in 2010 relative to 1996-1998, and 116% higher for *Salmonella* Newport (CDC 2011c). Typhimurium caused around 10,000 human infections reported to the CDC between 1987 and 1997, but only 4,983 laboratory-confirmed Typhimurium infections were reported in 2010 (CDC 2012a). The proportion of *Salmonella* causing infections in people that are resistant to at least one antimicrobial class has decreased between 2001 and 2010 (CDC, 2011c). Animal agriculture is a reservoir for *Salmonella;* therefore, many of the population changes and changes in AMR may be driven by changes in animal agriculture.

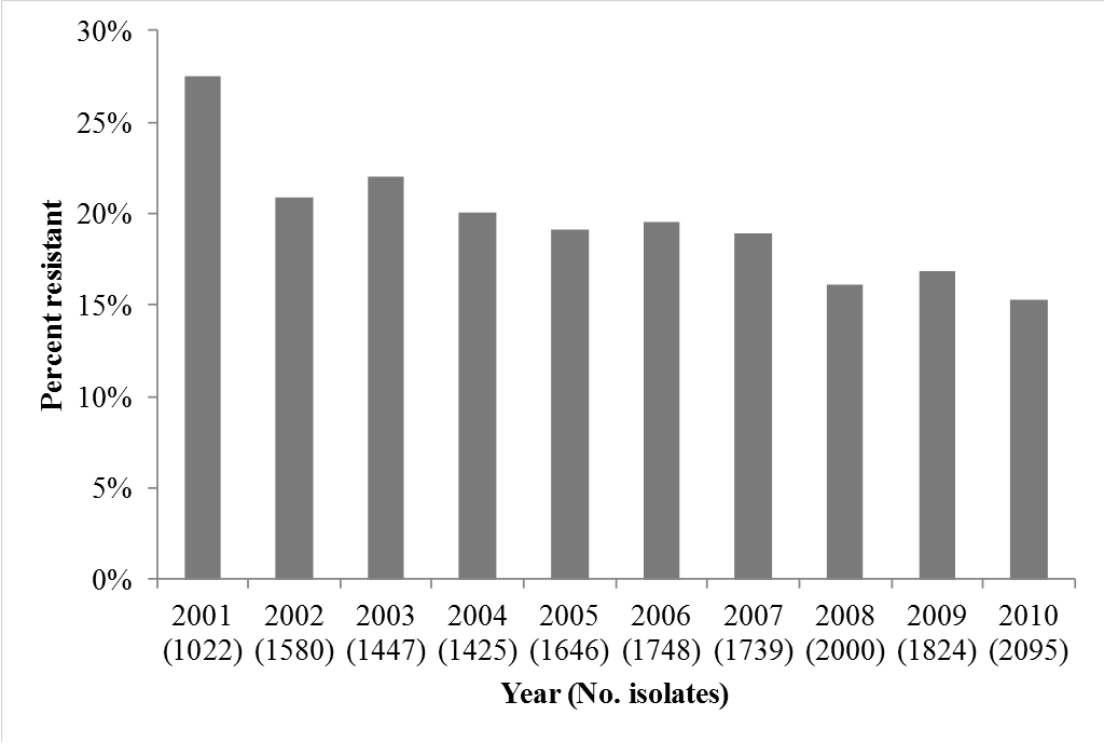

Figure 1 - Percent of isolates from laboratory-confirmed Salmonella infections in people that were resistant to at least one class of antimicrobial, 2001-2010.

Adapted from CDC, 2012 - National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Final Report, 2010. Atlanta, Georgia: U.S. Department of Health and Human Services

Trends in Antimicrobial Resistance of *Salmonella* from Dairy Cattle

Large changes in the prevalence and/or frequency of AMR have been noted in studies by NARMS and NAHMS. The directions of the changes in these two national surveillance systems, however, are conflicting. Between 1997 and 2006, there were substantial increases in the proportion of *Salmonella* isolates from clinically ill dairy cattle that were resistant, particularly for the antimicrobials amoxicillin/clavulanic acid, cefoxitin, ceftiofur, ceftriaxone and chloramphenicol (FDA 2006). The frequency of resistance to some antimicrobials, however, was

already high or remained unchanged (FDA 2010). Meanwhile, the prevalence estimates for AMR in *Salmonella* recovered from dairy farms decreased between 2002 and 2007 (USDA 2011b). These discordant findings are likely the result of differences in the sampling methodology, and highlight epidemiological differences between surveillance and clinical isolates of *Salmonella* from dairy cattle. There have nonetheless been important changes in the population of *Salmonella* within the United States. Changes in the population of *Salmonella* have coincided with changes in AMR. Increases in the proportion of *Salmonella* that are Typhimurium or Newport has caused increases in the frequency of resistance to chloramphenicol and cephalosporins, respectively (FDA 2011; Davis et al. 1999). Resistance genes that were once specific to these clonal strains, however, are now found in other *Salmonella* serotypes (Alcaine et al. 2005). Resistance patterns are frequently associated with a clonal strain, are not specific to that strain. Among clinical isolates from humans, 36% of the isolates with the ACSSuT phenotype were not DT104, and 33% of the isolates with the AmpC phenotype were serotypes other than Newport (CDC 2012c). Cephalosporin resistance has emerged within multiple serotypes of *Salmonella*, likely as a result of independent acquisitions of the bla-CMY-2 gene (S D Alcaine et al. 2005). Surveillance isolates of *Salmonella* Montevideo and Reading bearing variations of the AmpC phenotype were recovered in the most recent NAHMS study. Prior research has also shown independent acquisitions of bla-CMY2 within a single PFGE clade (Adhikari et al. 2010). Changes in the AMR of *Salmonella* in dairy cattle reflect the emergence of MDR strains, as well as the incorporation of resistance genes into multiple serotypes.

The emergence of novel strains of *Salmonella* may be a result of incorporation of AMR, virulence, or other fitness genes that allow it to fill a particular microbial niche or opportunistically invade susceptible populations. Examples of the emergence and dissemination

of novel *Salmonella* in livestock and poultry with important public health impacts include *Salmonella* Typhimurium DT104, MDR *Salmonella* Newport, and *Salmonella* Enteritidis. *Salmonella* Typhimurium DT104 was first recovered in the 1980's from cattle in the U.K, and was subsequently rapidly globally disseminated (Threlfall 2000). Characteristics of the strain are relatively homogeneous regardless of the geographic origin of the isolate. It is characterized by a distinctive PFGE pattern and the ACSSuT phenotype, which is chromosomally encoded by gene cassettes within integrons of *Salmonella* genomic island-1 (Threlfall et al. 2005). The emergence of DT104 in the 1990's in the U.S. caused increases in the prevalence estimates of AMR for certain antimicrobials, particularly chloramphenicol (Threlfall et al. 2006). MDR *Salmonella* Newport was initially reported after a description of the transmission of a ceftriaxone-resistant *Salmonella* strain from cattle to a child (Fey et al. 2000). This strain is characterized by penta-resistance (ACSSuT) plus resistance to extended-spectrum cephalosporins, encoded by the plasmid-associated *bla*-CMY2 gene. The proportion of *Salmonella* resistant to ceftiofur increased between the years 1999 and 2006 (FDA, 2010), and these increases were largely the result of the dissemination of MDR *Salmonella* Newport. A comparison of historic and contemporary isolates of *Salmonella* Newport highlighted the epidemiologic differences for the emergent strain of Newport (Berge et al. 2004) relative to the pansusceptible strain.

The primary mechanism by which regional or national level prevalence estimates of AMR in *Salmonella* change is through the emergence and dissemination of clonal subtypes (Butaye et al. 2006; Davis et al. 2002; Threlfall 2000). Clear temporal patterns of clonal displacement have been documented in the literature. Butaye et al., 2006 described succession of epidemic Typhimurium phage types in the UK, from DT204, DT204c, and DT104. The emergence of *Salmonella* Typhimurium DT104 was associated reduced recovery of

chloramphenicol-susceptible *Salmonella* Typhimurium strains, and increased recovery of choloramphenicol-resistant *Salmonella,* while the total number of *Salmonella* recovered remained relatively constant (Davis et al., 1999), and other phage types of Typhimurium recovered from cattle in the Netherlands (Duijkeren et al. 2002; Rabsch et al. 2001). Wiesner et al., 2009 showed that there as a temporal pattern of displacement of *Salmonella* Typhimurium ST 19 with other ST's that differed by a single base pair. The expansion in the population of *Salmonella* Enteritidis in poultry was associated with eradication campaigns against *Salmonella* Pullorum and Gallinarum. In an article from *Nature*, authors suggested that *Salmonella* Enteritidis filled an ecological void vacated by *Salmonella* Pullorum and Gallinarum (Bäumler et al. 2000). The emergence and dissemination of *Salmonella* clones has been followed by declines in the prevalence. Specifically, the proportion of cases caused by *Salmonella* Typhimurium DT104 has declined in recent years. After the frequency of illnesses caused by DT104 peaked in the 2000's, more recent evidence indicates that this strain is becoming less prevalent (40, 41). Threlfall et al., 2006, showed that a decline in the AMR of *Salmonella* Typhimurium recovered from clinically ill patients in the UK was caused by a concurrent decline in the proportion of *Salmonella* Typhimurium infections that were DT104 (Threlfall et al. 2006).

Changes in the population of *Salmonella* on Dairy Farms

The proportion of cows and operations that were positive for *Salmonella* increased for each of the cross-sectional studies done in 1996, 2002, and 2007 (USDA 2011b). Serotypes Meleagridis, Montevideo, and Mbandaka were the most commonly recovered isolates in all three study years (1996, 2002, and 2007) (USDA 2011b). In the NAHMS Dairy 2002 study, serogroup E1 serotypes comprised approximately 30% of the total number of *Salmonella* isolates recovered, compared to only 15% in 2007 (USDA 2011b). The percentage of farms where

serogroup E1 was recovered was 19% and 15% in 2002 and 2007 dairy studies, respectively (USDA 2011b). Additionally, *Salmonella* Cerro and *Salmonella* Kentucky represented a much larger proportion of the total *Salmonella* in 2007 relative to 2002 or 1996. Other researchers have reported on the expansion of the *Salmonella.* Cerro serotype in the Northeastern U.S, which primarily represents a single PFGE band pattern, lacks the *spvA* virulence gene, and rarely causes human disease (Hoelzer et al. 2011; Cummings et al. 2010). These studies demonstrate that clonal expansion may occur within the dairy population without being reflected in the population of *Salmonella* causing disease in humans. Emergent clones within the dairy population, however, may evolve to have important impacts on human health (Cummings et al. 2010). NARMS also reports on the population and AMR of *Salmonella* recovered from swabs of carcasses at slaughter, diagnostic cattle specimens and retail meats. The proportion of samples that are positive at slaughter has been consistently low (~1%) (USDA 2008). The most common serotypes recovered in the NARMS system in 2010 were Montevideo, Dublin, Kentucky, Anatum, and Typhimurium (USDA 2012a). The proportion of isolates that were identified as *Salmonella* Newport began peaked in 2003, just as the proportion of isolates that were identified as Montevideo declined. Since the increase of Newport and decline of Montevideo in 2003, the proportion isolates that were Montevideo increased every year except between 2009 and 2010, and it has been the most common serotype recovered in the NARMS surveillance system since 2004.

The rise and fall of epidemic clones may in large part be due to changes in the organism or the emergence of epidemic clones with higher fitness. However, widely distributed changes on livestock farms, including increases in herd sizes or the adoption of new technologies may alter the microbial environment and result in population changes. Specifically, use of antimicrobials,

particularly those in the feed which are dispersed to large numbers of animals on the farm, may allow AMR *Salmonella* to disseminate more freely. Changes in the management of dairy cattle, as well as consolidation of farms may result in altered host susceptibility and higher rates of transmission (Gardner et al. 2007). Simultaneous increases in dairy herd sizes and increases in the prevalence of *Salmonella* on dairy farms may not be coincidental (USDA 2011b; USDA 2009).

Temporal changes in prevalence or the population of *Salmonella* within dairy farms may be slow to occur. Over a year of sampling, 11 herds had greater than 10% prevalence on at least 2 of 5 visits, and a single serogroup represented the majority of the isolates (Fossler et al. 2004) . Pulsotypes of *Salmonella* causing clinical disease in dairy cattle were repeatedly recovered across sampling visits in a shorter term longitudinal study (Soyer et al. 2010). Previous research shows that substantial changes in the prevalence and AMR of *Salmonella* do not occur frequently. Thus, short-term longitudinal studies are often insufficient to document large shifts in the population within farms. A gradual shift in the population within a single dairy herd was documented over a two-year period, where *Salmonella* Cerro supplanted *Salmonella* Kentucky (Van Kessel et al. 2012). Because shifts in population often drive changes in AMR, longer-term longitudinal studies are necessary to investigate previously documented regional and national-level changes in the prevalence of *Salmonella* and the prevalence of AMR.

Conclusions

Substantial changes in the prevalence and AMR of *Salmonella* have been documented at the regional and national level. Studies using *Salmonella* recovered from diagnostic specimens have shown that changes in AMR of *Salmonella* occur through combinations of horizontal gene

transfer and dissemination of clonal MDR strains. However, there are few assessments of long-term changes in the population of *Salmonella* within dairy farms. Improved understanding of the frequency and drivers of within-farm changes may eventually enable more accurate identification of effective preharvest food safety practices.

CHAPTER TWO

Changes in the Antimicrobial Resistance Profiles of *Salmonella* Isolated From the Same Michigan Dairy Farms in 2000 and 2009

Structured Abstract

Objective: The objective of this study was to understand the type and distribution of changes in AMR in *Salmonella* within farms between 2000 and 2009.

Design: Retro-prospective

Sample Population: Eighteen Michigan dairy farms in 2000-2001 and 2009.

Procedure: Fecal samples from cows, calves, and environmental samples were taken on Michigan dairy farms in 2000-2001 and 2009. *Salmonella* were recovered from samples using tetrathionate enrichment and selective media. The minimum inhibitory concentration (MIC) was determined for 15 antimicrobials using the broth microdilution method. Multinomial, multilevel models were constructed to estimate the differences in MICs between years.

Results: The MICs of most antimicrobials were significantly lower in 2009 than in 2000, but were higher for amikacin and gentamicin. Decreases in MICs were in part due to changes in the prevalence of multidrug resistant strains, but were also distributed across the susceptible population of isolates. The type and direction of within-farm changes in MICs were similar for the majority of farms. These results suggest a decrease in antimicrobial resistance (AMR) and/or a change in the population structure of *Salmonella* that colonize dairy farms in Michigan.

Introduction

Salmonella is a worldwide cause of foodborne illness in people and livestock. Recent data from the Centers for Disease Control show that *Salmonella* is the leading cause of foodborne hospitalizations and death (Scallan 2011). Persons with suboptimal immune systems, particularly children, are most vulnerable to severe infections (CDC 2011c). AMR in *Salmonella* impairs the ability of physicians and veterinarians to treat serious infections. Patients infected with resistant strains of bacteria have higher hospital costs, a greater likelihood of septicemia, and higher mortality than patients infected with susceptible strains (Maragakis et al. 2008; Varma et al. 2005a)

Dairy farms serve as reservoirs of AMR *Salmonella* which can be transmitted to people through food vehicles or direct contact with animals (Fey et al. 2000). Beef and dairy products account for a substantial proportion of traceable *Salmonella* outbreaks (Lynch et al. 2006). The serotypes and molecular subtypes of AMR *Salmonella* isolated from dairy farms have significant overlaps with those that cause disease in humans (Alcaine et al. 2006; Soyer et al. 2010). Furthermore, resistant *Salmonella* strains may serve as donors of resistance genes to other pathogenic bacteria (Oppegaard et al. 2001). A study of *Salmonella* shedding on dairy farms conducted from 2000-2001 found that 27% of dairy farms harbored one or more AMR *Salmonella* (Ray et al. 2007). Changes in the prevalence of AMR *Salmonella* on dairy farms may have important impacts on human health. Monitoring systems of AMR in the U.S. have shown substantial changes in the types and frequency of resistance in *Salmonella* over the past ten years. The National Antimicrobial Monitoring System (NARMS) has shown increases in the frequency of cephalosporin-resistant *Salmonella* in clinically ill cattle (FDA 2010). Consecutive cross-sectional studies by the NAHMS (NAHMS) have shown a decrease in the prevalence of

AMR *Salmonella*. Approximately 12% of isolates were resistant to at least one antibiotic in 2002, compared to only 1.7% of isolates in 2007(USDA 2011b). Because of different sampling methodologies, the populations of *Salmonella* in the two surveillance systems are very different, and likely account for the discordant results between NAHMS and NARMS. Nonetheless, both monitoring systems suggest that the population of *Salmonella* on dairy farms and/or the AMR of those organisms has shifted significantly.

Rapid increases in AMR prevalence within farms can occur as a result of the introduction of resistant *Salmonella* strains, as exemplified by the clonal dissemination of *Salmonella* Typhimurium DT104 and MDR AmpC *Salmonella* Newport (Butaye et al. 2006). Changes in AMR may also occur due to the divergence of strain lineages as a result of horizontal gene transfer and genetic recombination (Sangal et al. 2010). Changes in AMR prevalence estimates identified by U.S. AMR monitoring systems could have important impacts on public health. However, it is unknown if these changes were uniformly distributed across farms, or were unevenly distributed, and dependent on farm characteristics. Improved understanding of the within-farm changes across years will provide additional insights into the epidemiology and ecology of AMR and *Salmonella* on dairy farms.

The objective of this study was to compare the AMR profiles of *Salmonella* isolates from the same farms at time points ten years apart. The hypothesis tested was that the AMR of *Salmonella* isolates within Michigan dairy farms decreased between the years 2000 and 2009.

Materials and Methods

This study used a retro-prospective study design to identify changes in the AMR profile within Michigan dairy farms. The data for this study consists of two components: retrospective

data collected from Michigan dairy farms in the year 2000, and prospective data collected 10 years later from the same Michigan dairy farms. Retrospective data were retrieved from a 2000-2001 multi-center, longitudinal study of *Salmonella* shedding on randomly selected dairy farms in Michigan, New York, Wisconsin, and Minnesota (Fossler et al. 2004). Stored *Salmonella* isolates collected in 2000 were retrieved from the Center for Comparative Epidemiology (CCE) at Michigan State University. Samples from the same farms were collected in August of 2009.

Farm Selection

For data collected in 2000, the number of farms sampled in each state was based on a sample size calculation with the following assumptions: 30% of the farms would be positive for *Salmonella*, a power 0.80, alpha of 0.10, and 2:1 ratio of exposed and unexposed farms for the risk factors of interest. In 2000, 31 dairy farms in Michigan were selected that met the following criteria: less than 100 miles from Michigan State University, milking greater than 30 Holstein cows, raising their own calves for replacements, and shipping milk year-round. For the data collected in 2009, all Michigan dairy farms that participated in 2000 were recruited.

Sample Collection

For the purposes of this manuscript, the word “sample” is used to refer to either animal fecal samples or environmental swabs collected from dairy farms. Comparable sampling plans for collecting fecal and environmental samples were used in both 2000 and 2009. In 2000, farms were sampled every other month, resulting in five sampling events. In 2009, four farms were sampled once, and two farms were sampled twice. Farms were sampled twice if the farm was negative for *Salmonella* on the first round of sampling, and had a greater than three percent shedding prevalence in 2000. Fecal samples were collected from the rectum of dairy cattle using

a single use rectal sleeve, and from calves using digital rectal retrieval. In both 2000 and 2009, healthy lactating cows and "target" animals were sampled from each farm. Target animals were defined as dairy animals most likely to be shedding *Salmonella*, including pre-weaned calves, cows identified as sick by the farm management, cows within 14 days of their calving date, and cows scheduled to be culled within 14 days. Target animals were preferentially sampled to increase the number of *Salmonella* isolates recovered and most accurately define the distribution of AMR in *Salmonella* within each farm. The number of samples collected was calculated to provide a 95% probability of recovering at least one *Salmonella* positive sample, assuming a prevalence of shedding of 9%. Similar sample size calculations for fecal and environmental samples were used in 2000 and 2009, and have been previously described (Fossler et al. 2004). Systematic sampling was used to obtain a representative sample of healthy cows and target animals. Environmental samples were taken using gauze swabs soaked with double-strength skim milk. Samples were taken from cow environments, including the maternity pen, sick pen, cull cow hide, milk filter, and manure storage area. Samples from calf environments included a composite sample from multiple calf pens. All samples were stored in commercial bags[1], placed in a cooler with ice, and submitted to the microbial epidemiology laboratory the following day.

Salmonella Isolation

Isolation of *Salmonella* was performed in the same laboratory with highly similar protocols in 2000 and 2009. With the exception of a confirmatory step (urea agar used in 2009), and the number of colonies chosen for confirmatory steps (five in 2009, and two in 2000), the protocols for the isolation and confirmation of *Salmonella* from fecal and environmental samples were identical. Samples were enriched by adding tetrathionate broth as to achieve a 1:10 dilution and incubating for 48 h at 37°C. The enriched sample was streaked onto XLT4 agar and

incubated for 24 h at 37°C. In 2009, up to five suspect colonies from XLT4 agar, (red or yellow with black centers) were inoculated onto TSI and urea agar slants, and incubated for 24 h at 37°C. In 2000, up to two suspect colonies from XLT4 agar, (red or yellow with black centers) were inoculated onto TSI only, and incubated for 24 h at 37°C. Colonies with test results typical for *Salmonella* (alkaline/acid/H2S positive, urease negative) were then inoculated onto lysine-iron agar and Simmons citrate agar slants. Colonies that were lysine decarboxylase and hydrogen sulfide positive in lysine-iron agar (purple slant, purple-black butt) as well as positive in Simmons citrate (blue) were considered positive for *Salmonella*. *Salmonella* isolates harvested in 2000 were frozen in tryptic soy broth/glycerol solution at -80 C and stored in cryovials. In 2009, these were retrieved, and underwent further biochemical confirmation before antimicrobial susceptibility testing. Isolates were stabbed onto a TSA slant, and kept at room temperature for a short period prior to antimicrobial susceptibility testing.

Antimicrobial Resistance Testing

To enable comparisons of AMR across years, *Salmonella* isolates collected in the 2000 study were tested concurrently with the 2009 isolates using the same commercially prepared microbroth dilution antimicrobial panels.[2] This panel contained a prepared range of concentrations for the following 15 antimicrobials: amikacin, amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. The tested antimicrobials were those used by NARMS (FDA 2010), and are considered to be critically important (amikacin, ampicillin, amoxicillin-clavulanic acid, ceftriaxone, ciprofloxacin, gentamicin, nalidixic acid, and streptomycin) or highly important (kanamycin, chloramphenicol, cefoxitin, sulfisoxazole, tetracycline, and trimethoprim-

sulfamethoxazole,) by the World Health Organization (WHO 2007). Quality control tests were performed using *E. coli* ATCC 25922 for all panels, and were all within acceptable limits. Colonies identified as *Salmonella* were streaked to Mueller Hinton agar and incubated for 18–24 hours at 37C. Testing was performed according to the instructions from the manufacturer of the automated microbroth dilution system (Trek Diagnostic Systems, Inc.), and panels were read with an autoreader. Breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) were used to classify isolates as susceptible, intermediate, or resistant (CLSI 2010). No CLSI interpretive criteria were available for ceftiofur or streptomycin, so breakpoints presented in the NARMS 2007 Annual Report were used (FDA 2010). Isolates that were classified as intermediate were considered to be sensitive for the purposes of analysis.

Statistical Analysis

Descriptive analyses were performed, including the tabulation of the MIC_{50}, $MIC_{90,}$ and the proportion of resistant isolates within years and within farms for each antimicrobial. For comparisons of the proportion of resistant isolates between groups, a chi-square test was used. In addition, a multinomial, multilevel, generalized linear mixed model was constructed for each antimicrobial tested using the GLIMMIX procedure in standard statistical software.[3] The levels of organization in the data include isolate, sample, farm, and year. Isolate-level analysis was performed, but only 4 of the 15 models converged. Therefore, the isolate with the maximum MIC was used to represent each sample, and the analysis was conducted at the sample-level. To account for the interdependence of samples within farms, farm was included as a random effect in each model. To estimate the differences in MIC's between years, year was included as a fixed effect in each model. Sample-level effects considered for inclusion in the models included

treatment with antimicrobials in the two weeks prior to sampling, and whether the sample originated from a cow, cow environment, calf, or calf environment. Initially, differences in MICs between isolates recovered animals and the environment were tested. To improve model stability, samples originating from cows, cow environments, calves, and calf environments were collapsed into the two-level variable sample source (cow or calf). Sample source was a considered a potential confounder, and was included as a fixed effect in each model. P values less than .05 were considered statistically significant.

Results

Salmonella Shedding

Shedding of *Salmonella* was higher in 2009 than in 2000. Twelve percent (97/836) of the samples, and 10 of the 18 farms were positive for *Salmonella* in 2009. In 2000, 8 of the same 10 farms and 6% of animal or environmental samples (264/5358) were positive for *Salmonella*. Isolates were included in the analysis if greater than one isolate was available from each farm at each time point. Two of the eight farms had only one isolate in either 2000 or 2009, and were excluded. A total of 391 and 261 isolates were used for the analysis from six farms sampled in 2000 and 2009, respectively.

Changes in the Prevalence of Resistance

The proportion of isolates resistant to at least one antimicrobial decreased between 2000-2001 and 2009 ($p<.001$) (**Table 5**). Only two resistant isolates were found in 2009. For the six farms that were positive for *Salmonella* in both years, at least one resistant isolate was found in five of six farms in 2000, and two of six farms in 2009. MDR was more frequent in 2000 than in 2009. Greater than half (37/59) of the resistant isolates recovered in 2000 exhibited resistance to

five antimicrobials. In 2009, one isolate was resistant to a single antimicrobial, and the other isolate was resistant to two antimicrobials. The predominant MDR phenotypes included the ACSSuT (21) ,GKSSuT (16), and GSSuT (7). The majority (51/59) of the resistant isolates found in 2000 came from two farms. Therefore, the change in the prevalence of AMR was unevenly distributed, and the overall decrease in the prevalence of resistant isolates was primarily distributed across only two farms.

Changes in the Minimum Inhibitory Concentrations

There were overall changes in the MIC_{50} and MIC_{90} of some antimicrobials between the years 2000 and 2009. Notable changes in the MIC_{50} between the two time points include a one-dilution increase in the MIC_{50} for gentamicin, and a one-dilution decrease in the MIC_{50} for nalidixic acid, chloramphenicol, and sulfisoxazole (**Table 6**). For chloramphenicol, nalidixic acid, and sulfisoxazole, the overall changes in MIC_{50} occurred on at least four out of six farms (**Table 7**). The overall increase in the MIC_{50} for gentamicin occurred in three of the six farms. In contrast to changes in the prevalence of resistance, changes in the MICs were distributed more evenly, and similar changes in the MIC occurred on a majority of farms.

Multivariate Model

The multilevel model was used to examine the effect of year and sample-level variables on the probability of a higher MIC for each antimicrobial. The effect of year was estimated while controlling for the effect of sample source. There was very little variability in the MIC distributions of ceftriaxone, ciprofloxacin, kanamycin, and streptomycin: The lack of variation resulted in poor model stability, and effects were not estimated for ciprofloxacin, kanamycin, and

streptomycin. There was a somewhat larger amount of variability in the MIC distributions for amoxicillin-clavulanic acid, ampicillin, and tetracycline. For these antimicrobials, most of the isolates with different MICs also exhibited a MDR phenotype. Other antimicrobials, however, including amikacin, cefoxitin, ceftiofur, chloramphenicol, gentamicin, nalidixic acid, and sulfisoxazole, exhibited larger variability in the MIC distributions.

The multivariate model was sensitive to detection of differences in MICs between years. For all antimicrobials in which the model converged, there were significant differences between 2000 and 2009, except ceftriaxone. Based on the multivariate model, isolates from 2009 had a significantly lower MIC for amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, chloramphenicol, nalidixic acid, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole ($p<0.05$) (**Table 8**). By contrast, isolates from 2009 had a higher MIC for amikacin and gentamicin ($p<.005$). The MIC was not significantly different between years for ceftriaxone.

Sample level effects

In this study, the proportion of isolates resistant did not differ by class of adult cow (sick, fresh, close-up, or cull). Isolates from calves, however, were significantly more likely to be resistant (40/131) than isolates from healthy cows (7/309) ($p<.0001$), and frequently exhibited the ACSSuT phenotype (15/131). The MICs of animal isolates were not significantly different from environmental isolates for any antimicrobial. Therefore, animal/environment source and animal class were collapsed into the two level variable 'sample source' (cow or cow environment vs. calf or calf environment). Controlling for the effect of year, isolates from calves had a significantly higher MIC than isolates from cows for ampicillin, amoxicillin-clavulanic acid, sulfisoxazole, and tetracycline ($p<.005$) (**Table 8**). When the model was run without the MDR

isolates (n=37), the MICs for isolates from calves were not significantly different than cows for any antimicrobial. Thus, the differences in MICs between cows and calves were due to a greater frequency of penta-resistant *Salmonella* in calves, rather than changes in MICs that were distributed across both susceptible and resistant isolates.

Discussion

Observational studies of AMR on farms commonly use cross-sectional or retrospective study designs. Cross-sectional studies of AMR on dairy farms, while useful to generate prevalence estimates and identify risk factors, are unable to identify changes in AMR that occur within farms over time. While longitudinal studies of AMR on dairy farms have been conducted (Adhikari et al. 2009a; Ray et al. 2007), the relatively small temporal separation of sampling events may not allow for an analysis of changes in AMR that may occur over many years due to genetic divergence or broad changes in the population of *Salmonella*. Retrospective studies of contemporary and historic isolates have used strain collections accumulated in diagnostic laboratories (Berge et al. 2004; Harbottle et al. 2006). Collections of clinical isolates, however, represent a biased population of epidemiologically unrelated strains. Results from these studies cannot be extrapolated to the entire population of *Salmonella* on dairy farms, and cannot identify changes that occur within each farm. Repeated assessments of the within-farm AMR profiles at multiple time points would be useful to identify the type, magnitude, and distribution of the changes in AMR profiles within dairy farms.

Epidemiological studies of AMR most commonly use measures of prevalence that rely on interpretive criteria established by the Clinical Laboratory Standards Institute (CLSI). These breakpoints are chosen to reflect likely clinical outcomes of treatment, and are specific to the

host, bacteria species, antimicrobial, dose, and route. However, increases in the MIC that occur below the breakpoint have been found to be relevant to the outcome of treatment (D L Paterson et al. 2001; Sakoulas et al. 2004). Additionally, organisms classified as susceptible may still harbor important genetic determinants of resistance (Frye et al. 2010). Commonly used prevalence measures such as the percent of isolates resistant may not reflect relevant changes in the distribution of MICs on dairy farms. Dichotomization of the MIC data results in a biologically and a statistically detrimental loss of data. Therefore, the dependent variable for the multivariate model used to analyze these data was the multinomial MIC measurement generated by the Sensititre ® testing system.

The prevalence estimates for resistance described in this study must be interpreted with caution. The small number of farms used in this study limits the temporal and geographic inferential scope of the results. The smaller collection and number of sampling days in 2009 relative to 2000 may result in a collection of isolates that are less representative of the diversity of strains present in cattle and the environment. Also, the "point-in-time" nature of the sampling plans provides only a snapshot of the organisms that were present on the farms between 2000 and 2009, and prevalence estimates have been found to be variable within the same farm over time (Rostagno et al. 2011). Furthermore, differences in estimates of AMR could be due to a lack of precision associated with imperfect sampling plans, rather than true changes in the bacterial population (B. Wagner et al. 2003). These results are intended to understand the type and distribution of changes in MIC profiles within farms.

Nonetheless, the concurrent increase in shedding and decrease in AMR found in this study are in agreement with previous NAHMS studies, which used cross-sectional sampling of dairy farms (USDA 2011b). The results of this study also suggest that the observed changes

between 2000 and 2009 were not randomly distributed across dairy farms. Rather, similar within-farm changes in the MIC distributions occurred on a majority of farms (**Table 7**). Of the six farms tested at both time points, four or more farms showed a similar change in the within-farm MIC_{50} for cefoxitin, chloramphenicol, naladixic acid, and sulfisoxazole. These results, together with the results of the statistical model, suggest large differences between years, and smaller differences between farms from the same year. Similarities in the AMR between farms from the same time point suggest that the dissemination of clonal subtypes between farms is in part responsible for the observed changes in antimicrobial profiles. The subtypes of *Salmonella* with the highest ability to compete in dairy farms may have proliferated between 2000 and 2009. An ecological study that compared the AMR of mastitis pathogens in the United States with those in Denmark found relatively small within-country differences between organic and conventional farms compared to large differences in the AMR between countries (Sato et al. 2004). While the differences between each country are likely due to differences in the production systems, the homogeneity within each country relative to the differences between countries may be due to the movements of livestock, people, equipment, and wildlife. This concept may be manifested in our results by large differences between years, and smaller differences in AMR profiles between farms from the same year.

Some of the change in the distribution of MICs could be attributed to the change in prevalence of MDR *Salmonella.* Global or national level changes in the prevalence of AMR may be caused by the dissemination (or disappearance) of clonal MDR subtypes, including *Salmonella* Typhimurium DT104 and MDR Amp C *Salmonella* Newport (Butaye et al. 2006). Likewise, the prevalence of resistance in *Salmonella* at the farm level can rapidly change as result of the introduction or disappearance of novel resistant strains (Adhikari et al. 2009a). In

this study, changes in the farm-level prevalence of AMR were reflective of the detection or lack of detection of isolates with MDR phenotypes at either time point. In particular, ampicillin, amoxicillin-clavulanic acid, and tetracycline exhibited bimodal distributions, and the majority of the decrease in resistance between 2000 and 2009 was due the presence or absence of MDR organisms. MDR phenotypes found in 2000, including the phenotype associated with *Salmonella* Typhimurium DT104, were not found on the same farms in 2009. A decline in the frequency of *Salmonella* Typhimurium DT104 in diagnostic laboratories has been noted (Threlfall et al. 2005), but the most recent data from the NARMS did not indicate a clear trend in the proportion of *Salmonella* Typhimurium from cattle with the ACSSuT phenotype (CDC 2012c). In the NAHMS studies, the proportion of penta-resistant isolates was similar in 1996, 2002, and 2007. The 2007 study, however, was the first NAHMS study to fail to identify the ACSSuT phenotype within isolates of *Salmonella* Typhimurium (USDA 2011b). The decline in the prevalence of MDR phenotypes found in this study may be reflective of national-level or regional-level trends in the prevalence of MDR subtypes of *Salmonella*.

There were changes in the MIC distributions that occurred on a majority of farms that could not be attributed to the presence or absence of MDR organisms. There was substantial variability in the MIC distributions that occurred below the breakpoint for resistance. For instance, no isolates in this study were resistant to ceftiofur, and only one isolate was resistant to cefoxitin. Nonetheless, the 2000 and 2009 MIC frequency distributions for ceftiofur and cefoxitin were visibly different, and the model detected significant decreases in the MICs between the two years. These results suggest that there were changes in both the resistant and susceptible populations of *Salmonella.* The prevalence of MDR subtypes of *Salmonella* declined,

and there were also changes in the MIC distributions within the susceptible populations of *Salmonella.*

There are many potential explanations for the changes between 2000 and 2009. A possible explanation for the decline in resistance and increase in shedding is the displacement of less susceptible populations of *Salmonella* by strains with a higher level competitive fitness in the dairy farm niche. AMR genes confer an advantage when selective antimicrobial pressure if present, but often result in overall decreases in the competitive fitness of the associated strain (Zhang et al. 2006). Alternatively, compensatory changes in the genome can allow for persistence of resistant strains, regardless of antimicrobial selective pressure (Enne et al. 2004; Walk et al. 2007). The observed changes in the within-farm AMR profile may be a result of the dissemination and proliferation of *Salmonella* strains with different AMR profiles. Thus, changes may be explained by the simple displacement of one population of *Salmonella* by another. Conversely, genetic divergence of *Salmonella* strains within farms over time may result in changes in the susceptibility to antimicrobials. A recent phylogenetic analysis of a collection of *Salmonella* Newport concluded that mutations and homologous recombination resulted in divergent lineages (Sangal et al. 2010). The changes in AMR observed in this study may be a combination of both dissemination and an ongoing evolution of isolates within farms. Alcaine et al., 2005 concluded that the emergence of ceftiofur-resistant *Salmonella* was caused by "independent emergence followed by clonal spread."

Dairy farm production systems have undergone dramatic changes, including increases in herd sizes, between 2000 and 2009. In this study, four of the six herds were 70-100% larger (more lactating cows) in 2009 than in 2000. Prior studies have found that *Salmonella* are more likely to be isolated from larger herds than smaller herds. Decreases in the MICs occurred for

beta-lactam antimicrobials in spite of the selective pressure that is present on most dairy farms (USDA 2009). By contrast, the MICs for two aminoglycosides, including gentamicin and amikacin, increased despite a lower frequency of use than most beta-lactam antimicrobials (USDA 2009). The identified changes in aminoglycoside MICs may represent local or regional change, or may reflect the specific selective pressure present on these farms. Relevant changes that occurred on a majority of farms may have resulted in similar changes in the shedding prevalence and MIC profiles across farms.

Conclusions

This study used *Salmonella* isolates from the same farms collected at time points ten years apart to understand changes in the AMR profiles of *Salmonella* on dairy farms in Michigan, USA. The MICs of most antimicrobials were significantly lower in 2009 than in 2000, but were higher for amikacin and gentamicin. Decreases in MICs were in part due to changes in the prevalence of multidrug resistant strains, but were also distributed across the susceptible population of isolates. The type and direction of within-farm changes in MICs were similar for the majority of farms. Identification of the serotypes and molecular subtypes will be necessary to understand to what degree genetic divergence or dissemination of strains contributed to the changes in AMR within farms.

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[1] WhirlPak®, Nasco, Fort Atkinson, WI

[2] CMV1AGNF; Trek Diagnostic Systems, Inc

[3] SAS, Version 9.2, Cary, NC

Table 5 - Total number of *Salmonella* isolates and number of antimicrobial resistant *Salmonella* isolates recovered from Michigan dairy cattle in the years 2000-2001 and 2009.

Farm	2000			2009		
	Resistant[a]		Total[b]	Resistant[a]		Total[b]
101	23	(66)	35	0		103
111	2	(1.1)	190	0		17
114	5	(4.2)	117	0		47
121	0		3	1	(2.0)	48
125	1	(6.3)	16	1	(2.5)	40
129	28	(93)	30	0		6
Total	59	(15)	392	2	(0.8)	261

[a] Number (%) of isolates resistant to at least one antibiotic.

[b] Total number of isolates from each farm in each year.

Table 6 - MIC_{50} and MIC_{90} for *Salmonella* isolates recovered from Michigan dairy cattle in 2000-2001 and 2009.

Antimicrobial	MIC_{50}		MIC_{90}	
	2000-2001	2009	2000-2001	2009
Amikacin	1	1	2	2
Amox-clav	1	1	1	1
Ampicillin	1	1	1	1
Cefoxitin	2	2	4	4
Ceftiofur	1	1	1	1
Ceftriaxone	0.25	0.25	0.25	0.25
Chloramphenicol[a]	8	4	8	8
Ciprofloxacin	0.02	0.02	0.02	0.02
Gentamicin[a]	0.25	0.5	0.5	0.5
Kanamycin	8	8	8	8
Naladixic Acid[a]	4	2	4	4
Streptomycin[b]	32	32	64	32
Sulfisoxazole[a]	64	32	256	64
Tetracycline[b]	4	4	32	4
Trimeth-Sulfa[b]	0.12	0.12	0.25	0.12

[a] Different MIC_{50} values between 2000 and 2009

[b] Different MIC_{90} values between 2000 and 2009

MIC_{50} – minimum inhibitory concentration that inhibits the growth of 50% of the isolates

MIC_{90} – minimum inhibitory concentration that inhibits the growth of 90% of the isolates

Table 7 - Number of farms with an increase, decrease or no change in the MIC_{50} for *Salmonella* isolates recovered between 2000-2001 and 2009.

Antimicrobial	**Frequency of Farms**		
	Increase	**Decrease**	**No Change**
Amikacin	2	0	4
Amox-Clav	0	1	5
Ampicillin	0	1	5
Cefoxitin	0	4	2
Ceftiofur	1	1	4
Ceftriaxone	0	0	6
Choramphenicol[a]	0	4	2
Ciprofloxacin	0	0	6
Gentamicin	3	1	2
Kanamycin	0	1	5
Naladixic Acid[a]	0	4	2
Streptomycin	0	2	4
Sulfisoxazole[a]	0	5	1
Tetracycline	0	2	4
Trimeth-Sulfa	0	1	5

[a]There was also a corresponding overall change in the MIC_{50}

MIC_{50} – minimum inhibitory concentration that inhibits the growth for 50% of the isolates

Table 8 - The effect of 'year' and 'sample source' on the MIC from the multivariable model. Estimates reflect the probability of a higher MIC for each antimicrobial.

Antimicrobial	Year[a]		Sample Source[b]	
	Estimate	p value	Estimate	p value
Amikacin	1.17	<.001[c]	-0.02	0.953
Amox-clav	-1.95	0.002[c]	1.79	<.001[c]
Ampicillin	-1.51	0.003[c]	1.18	0.005[c]
Cefoxitin	-3.02	<.001[c]	-0.43	0.107
Ceftiofur	-1.89	<.001[c]	-0.27	0.428
Ceftriaxone	2.23	0.112	2.20	0.080
Chloramphenicol	-3.31	<.001[c]	0.33	0.260
Ciprofloxacin	Not estimated		Not estimated	
Gentamicin	0.91	0.002[c]	0.47	0.094
Kanamycin	Not estimated		Not estimated	
Naladixic Acid	-1.87	<.001[c]	-0.29	0.337
Streptomycin	Not estimated		Not estimated	
Sulfisoxazole	-1.82	<.001[c]	0.87	<.001[c]
Tetracycline	-3.25	0.004[c]	1.90	0.001[c]
Trimeth-Sulfa	-2.44	0.024[c]	0.41	0.325

[a]Isolates from 2009 relative to isolates from 2000 (controlling for sample source)
[b]Isolates from calves relative to isolates from cows (controlling for year)
[c]P value < .05

CHAPTER THREE

Changes in the Prevalence of *Salmonella* on Michigan Dairy Farms between 2000-2001 and 2009

Structured Abstract

Objectives: Determine the overall and within-farm changes in the prevalence of *Salmonella* between 2000-2001 and 2009 and the associations with changes in herd size and management practices.

Design: Retro-prospective

Sample Population: Eighteen Michigan dairy farms in 2000-2001 and 2009

Procedure: *Salmonella* isolates and data collected during a 2000-2001 study were retrieved for Michigan dairy herds. Farms were sampled prospectively in 2009, and comparable data were collected. The overall change in prevalence and the association with changes in herd size and management practices for 18 dairy farms was tested using a generalized linear mixed model.

Results:

Between 2000-2001 and 2009, the prevalence of *Salmonella* increased in adult cow and environmental samples, but not calf samples. Herds that increased in herd size between time frames had larger within-farm increases in the prevalence of *Salmonella*. Management practice changes, including increased use of liquid manure and grazing pastures where manure had recently been applied were significantly associated with increases or decreases in *Salmonella* prevalence.

Conclusions:

Within-farm increases in the prevalence of *Salmonella* among adult cows between 2000-2001 and 2009 were significantly associated with increases in herd size and changes in management practices.

Introduction

Globally, an estimated 93.8 million illnesses and 155,000 deaths are caused by non-typhoidal *Salmonella* annually (Majowicz et al. 2010). A recent report from the CDC states "*Salmonella* infection should be targeted because it has not declined significantly in more than a decade" (CDC 2011c).The incidence of salmonellosis in people was significantly higher in 2010 relative to 2006-2008 (CDC 2011c), and the "Healthy people 2010" target (6.28/100,000) for reducing the incidence of salmonellosis was not met.

Efforts to reduce the incidence or impact of *Salmonella* in humans will be enhanced by controlling the pathogen in livestock populations. Dairy farms harbor zoonotic *Salmonella* strains that are important causes of illness in people (Soyer et al. 2010; Hoelzer et al. 2010).There is significant overlap in the antimicrobial resistance (AMR) phenotypes, serotypes, and genetic subtypes of *Salmonella* found on dairy farms and those that cause disease in humans (Soyer et al. 2010; Alcaine et al. 2006).

In dairy cattle, *Salmonella* can cause asymptomatic colonization or clinical disease with wide ranges of severity. The organism can be recovered from 90% of dairy farms (Fossler et al. 2004), but the burden of *Salmonella* is not evenly or randomly distributed across herds (Fossler et al. 2004; USDA 2011b). Rather, large herds are more frequently positive and have a higher prevalence (Blau et al. 2005; Habing et al. 2012; Fossler et al. 2004), and a relatively small proportion of herds account for a majority of shedding (Wells et al. 2001). The distribution of *Salmonella* across herd types suggests that management practices influence the ability of *Salmonella* to colonize farms. In a large study of dairy farms in the Midwest and Northeast, *Salmonella* shedding was significantly associated with the use of liquid manure, grazing or

eating hay that had been contaminated with manure, lack of use of rumensin in heifers, and lack of protection of feed bins from birds Fossler et al. 2005a).

Another unique aspect of the epidemiology of *Salmonella* is its ability to persist within dairy herds for years (Van Kessel et al. 2007; Van Kessel et al. 2012). In a recent study, authors demonstrated the persistence of a single pulsotype within a dairy herd for over five years (Van Kessel et al. 2012). Eleven high prevalence herds in the Midwest or Northeast had >10% shedding for the duration of a 1-year longitudinal study (Fossler et al. 2004). Strains within indistinguishable PFGE banding patterns are repeatedly recovered across sampling visits (Soyer et al. 2010; Fossler et al. 2004). Slow changes in the population of *Salmonella* within farms may necessitate longer-term longitudinal study designs.

Furthermore, national-level prevalence estimates for *Salmonella* on dairy farms have increased substantially over time. Between cross-sectional studies done by the USDA in 1996, 2002, and 2007, the proportion of farms and cows that were positive for *Salmonella* roughly doubled (USDA 2011b). These increases may be associated with changes in the *Salmonella* population or changes in dairy farm characteristics and management practices. The objective of the following research was to use a long term approach to understand the association between within-farm prevalence changes and changes in dairy farm characteristics and management practices. The **hypothesis** tested by this retro-prospective study was that the prevalence of *Salmonella* on Michigan dairy farms increased between 2000-2001 and 2009, and is associated with herd size increases and/or management practice changes.

Materials and Methods

Study Design

Data for this retro-prospective study consists of two components: retrospective data retrieved from a study of *Salmonella* on Michigan dairy farms, and data collected prospectively 10 years later from the same Michigan dairy farms. Retrospective data were retrieved from a longitudinal study of *Salmonella* shedding on randomly selected dairy farms in Michigan, New York, Wisconsin, and Minnesota (Fossler et al. 2004). Stored *Salmonella* isolates collected from Michigan dairy farms between June 2000 and September 2001 were retrieved from the Center for Comparative Epidemiology (CCE) at Michigan State University. Samples from the same farms were collected prospectively in July and August of 2009.

Farm Selection

In 2000-2001, 31 dairy farms in Michigan were randomly sampled that met the following criteria: less than 100 miles from Michigan State University, milking greater than 30 Holstein cows, raising their own calves for replacements, and shipping milk year-round. For the data collected in 2009, all Michigan dairy farms that participated in 2000-2001were recruited.

Sample collection

For the purposes of this study, the word “sample” is used to refer to either animal fecal samples or environmental swabs collected from dairy farms. Comparable sampling plans for collecting fecal and environmental samples were used in both 2000-2001 and 2009. Two herds (101,102) were initially sampled weekly for 8 consecutive visits in the spring/summer of 2000, and then subsequently sampled every two months for five consecutive visits. Other Michigan herds in the study were sampled every two months for five consecutive visits beginning in the

fall of 2000. In 2009, farms were sampled once, and sampled twice if the farm was negative for *Salmonella* on the first round of sampling, and had a greater than three percent shedding prevalence in 2000-2001. Fecal samples were collected from the rectum of dairy cattle using a single use rectal sleeve, and from calves using digital rectal retrieval. In both 2000-2001 and 2009, healthy lactating cows and "target" animals were sampled from each farm. Target animals were defined as dairy animals most likely to be shedding *Salmonella,* including pre-weaned calves, cows identified as sick by the farm management, cows within 14 days of their calving date, and cows scheduled to be culled within 14 days. Target animals were preferentially sampled to increase the number of *Salmonella* isolates recovered and most accurately define the population of *Salmonella* within each farm. The number of samples collected was calculated to provide a 95% probability of recovering at least one *Salmonella* positive sample, assuming at least 9% of the cattle were shedding the organism. Similar sample size calculations for fecal and environmental samples were used in 2000 and 2009, and have been previously described (Fossler, et al., 2004). Systematic sampling was used to obtain a representative sample of healthy cows and target animals. Environmental samples were taken using gauze swabs soaked with double-strength skim milk. Samples were taken from cow environments, including the maternity pen, sick pen, cull cow hide, milk filter, and manure storage area. Samples from calf environments included a composite sample from multiple calf pens. All samples were stored in commercial bags, placed in a cooler with ice, and submitted to the Microbial Epidemiology Laboratory at the Center for Comparative Epidemiology the following day.

Salmonella Isolation

Isolation of *Salmonella* was performed in the same laboratory with highly similar protocols in 2000 and 2009. With the exception of a confirmatory step (urea agar used in 2009),

and the number of colonies chosen for confirmatory steps (five in 2009, and two in 2000), the protocols for the isolation and confirmation of *Salmonella* from fecal and environmental samples were identical. Samples were enriched by adding tetrathionate broth to achieve a 1:10 dilution and incubating for 48 h at 37°C. The enriched sample was streaked onto XLT4 agar and incubated for 24 h at 37°C. In 2009, up to five suspect colonies from XLT4 agar, (red or yellow with black centers) were inoculated onto TSI and urea agar slants, and incubated for 24 h at 37°C. In 2000, up to two suspect colonies from XLT4 agar, (red or yellow with black centers) were inoculated onto TSI only, and incubated for 24 h at 37°C. Colonies with test results typical for *Salmonella* (alkaline/acid/H2S positive, urease negative) were then inoculated onto lysine-iron agar and Simmons citrate agar slants. Colonies that were lysine decarboxylase and hydrogen sulfide positive in lysine-iron agar (purple slant, purple-black butt) as well as positive in Simmons citrate (blue) were considered positive for *Salmonella*. *Salmonella* isolates harvested in 2000 were frozen in tryptic soy broth/glycerol solution at -80 C and stored in cryovials. In 2009, these were retrieved, and underwent further biochemical confirmation before antimicrobial susceptibility testing.

Statistical Analysis

Prior research indicated a strong seasonal correlation with the farm prevalence of *Salmonella* (Fossler et al. 2004). To increase the validity of comparisons of prevalence between the two time periods, the summer sampling visit from 2000-2001 that most closely matched the summer sampling date from the same farm in 2009 were included in the statistical analysis. Cow or calf samples were not taken in the summer months (July, August, or September) for two herds (102 and 131), so samples from June 2001 were used instead. Calves were not being raised on one farm in 2009 (102), and this farm was not included in the analysis for calf shedding.

Fisher's exact tests were used to compare within-farm differences in the proportion of samples positive between 2000-2001 and 2009. Separate generalized linear mixed models (PROC GLIMMIX, SAS, v. 9.2, Cary, NC) were used to analyze overall differences in the summer prevalence of cow, calf and environmental samples between 2000 or 2001 and 2009. The outcome was the number of positive samples/total samples, and fixed effects included year (2000-2001 or 2009) and herd size (total lactating cows). A random intercept was specified for the herd.

Herd-level management practices previously associated with *Salmonella* shedding in cows (Fossler et al. 2005a) were considered for inclusion in the generalized linear mixed model. For shedding in cows, they included eating or grazing roughage from fields where manure was applied in solid or liquid form, surface application of slurry on owned or rented land, not storing purchased concentrates and protein feeds in an enclosed building, and the lack of use of rumensin in weaned calves or heifers. For calves, examined management practices included housing sick cows in the maternity pen and lack of usage of antimicrobials in the milk replacer.

To test the hypothesis that changes in management practices were associated with the magnitude of within-farm prevalence differences between 2009 and 2000-2001, year (2000-2001 or 2009), management practice change, and the interaction between year and management practice change were included in the model. Due to the low number of farms, these models did not converge, and instead a management practice change index was creating by subtracting the 2009 binary value of each management practice (1 or 0) from the 2000-2001 value and summing across the four management practice variables, resulting in a variable with a possible range of -4 to 4. The interaction between year and management practice change index was included in the

model to determine the association with the magnitude of the difference in prevalence estimates between years.

Model effects were estimated using maximum likelihood estimation, and overall significance of the fixed effects and random intercept were tested by comparing -2LogL between full and nested models using the likelihood ratio test. Assumptions of the model were checked by examining the distribution of herd-level residuals (EBLUP's). Collinearity of the fixed effects in the model (year, herd size, and change index) was assessed using the Pearson correlation coefficient.

Results

Changes in Prevalence across Sampling Visits, 2000-2001

In 2000-2001, 5,358 cow, calf, or environmental samples were collected from 18 different Michigan dairy farms. Two herds (101,102) were sampled weekly for 8 consecutive visits followed by 5 consecutive bimonthly visits. The remaining 16 herds were sampled bimonthly for 5 consecutive visits. Eleven, four, and five herds had at least one positive cow, calf, and environmental sample, respectively. Over all samples and herds, *Salmonella* was recovered from 6% (264/5,358) of samples, and 77% (14/18) of herds (**Table 9**). Within herds, the overall prevalence (all visits and all samples) ranged from 0% to 45%. The prevalence was 20% to 45% for two herds and <10% for 12 herds. Two high prevalence herds had >20% prevalence on all but one of the five visits, and the prevalence ranged between 13% and 62%. These two herds accounted for 73% of the positive samples during the time frame. Of the 14 positive herds, nine had fewer than three positive samples over five sampling visits. During 2000-2001, the majority of herds were either consistently positive or had a very low prevalence

of *Salmonella*. For most sample types, the proportion of samples positive was higher in the summer visits relative to visits during other seasons. Therefore, seasonally matched visits were used to make comparisons between years.

Three of the fourteen positive herds had important changes in the prevalence of subtypes across the 2000-2001 sampling visits. On farm 129, two negative sampling visits in the winter and spring of 2001 were followed by recovery of MDR *Salmonella* Senftenberg in cow and calf areas on two subsequent summer visits. On farm 101, MDR *Salmonella* Typhimurium was recovered first from two adult cows, and then recovered from 4/6 preweaned calves on 3 subsequent weekly visits at the outset of the study. However, these strains were not recovered on the five subsequent bimonthly visits. Farm 125 was negative on four consecutive visits, and then 27% (13/48) of the cow samples were positive for a pansusceptible strain *Salmonella* on the last visit in the summer of 2001.

Prevalence in 2009

In the summer of 2009, 830 samples were collected from the same 18 Michigan dairy farms using similar sampling schemes (**Table 9**). At least one isolate was recovered from 10 herds, and the number of herds with at least one positive cow, calf, and environmental sample was 9, 4, and 10, respectively. The within-farm prevalence over all sample types ranged from 0% to 63%, and was over 50% for 2 herds, between 20% and 50% for 3 herds, and less than 10% for 13 herds.

Within-herd Differences in Prevalence across Seasonally-matched Visits, 2000-2001 and 2009

Eleven of the eighteen herds had less than 10% prevalence in both summers, including three farms where *Salmonella* was not recovered in either year (**Table 13**). For the high

prevalence herds in 2000-2001 (Farms 111 and 114), one was negative in 2009, and one had a >10% prevalence in 2009. A Fisher's exact test was used to compare the prevalence of *Salmonella* in cow, calf, and environmental samples between summers of 2000 or 2001 and 2009. The within-farm cow prevalence was significantly higher ($p < 0.05$) in 2009 for two farms, and significantly lower in 2009 for one farm (**Table 12**). The proportion of calf samples was significantly lower in 2009 for one herd, and the proportion of environmental samples was significantly higher for two herds in 2009 relative to 2000 or 2001 (**Table 12**).

Shedding in Cows

A generalized linear mixed model and seasonally matched summer sampling visits were used to test the hypothesis that the overall prevalence of *Salmonella* in cow, calf, or environmental samples had changed between the summers of 2000-2001 and 2009. The likelihood ratio test of the random effect for farm in the mixed model for cows was significant, suggesting that the prevalence of *Salmonella* in 2009 was not statistically independent of the prevalence in the same herd in 2000-2001 ($p<0.001$).

Salmonella was recovered from 9% (60/641) and 13% (64/468) of cow samples in summers of 2000 or 2001 and 2009, respectively (**Table 9**). Cow samples from 2009 had twice the odds of being positive relative to a cow sample from 2000-2001 (95% CI: 1.21 - 3.09). However, the difference in prevalence between years was not significant after including herd size in the model (**Table 10**). Higher prevalence of *Salmonella* in cow samples was significantly associated with larger herd sizes (**Table 10**). Herds that underwent larger increases in herd size between the two time points had larger increases in the prevalence of *Salmonella* in cow samples between time frames. Of three herds with significant within-farm increases in the prevalence of

Salmonella in cow or environmental samples (Farms 109, 101, and 111), 2 had increased the number of lactating cows on the farm by at least 200 cows (**Figure 2**).

Management practices examined included those that were found to be significantly associated with *Salmonella* in a prior longitudinal study of *Salmonella* shedding on these herds (**Table 11**) (Fossler et al. 2005a; Fossler et al. 2005b). Adjusting for herd size in each time period, herds that adopted a greater number of management practices previously associated with *Salmonella* shedding in cows had larger positive differences in the cow prevalence between 2009 and 2000-2001 ($p<0.001$). Two herds with the largest increase and decrease in prevalence (109 and 111, respectively) reported changes in management practices consistent with the previously reported effects on *Salmonella* shedding. Herd 109 reported cows eating or grazing roughage where manure had been applied, and surface application of liquid manure in 2009; however, neither of those practices were used in 2000-2001 (**Table 11**). By contrast, the farm with the largest decrease in prevalence (Farm 111) between 2000 or 2001 and 2009 reported storing purchased concentrates in an enclosed building and feeding rumensin in weaned calves or heifers in 2009, whereas neither of those practices was used in summer, 2001. Based on previous research (Fossler et al. 2005), starting these 2 practices would be expected to decrease *Salmonella* shedding.

Model results for cow shedding suggest that overdispersion may be significantly affecting the results. The ratio of chi-square residuals/degrees of freedom was >3 in each model suggesting inadequate fit and overdispersion in the data. Future analyses may use model techniques that correct for inaccurate standard error estimates in overdispersed data, or use generalized estimating equations with robust standard errors.

Shedding in Calves

For calves, *Salmonella* was recovered from 8% (12/145) and 7% (11/163) of samples from 2000-2001 and 2009, respectively (**Table 9**). Adjusting for the non-independence of samples within herds, the proportion of samples positive for *Salmonella* in calves was not significantly different (**Table 10**). Management practices changes, including changes in feeding medicated milk replacer and the use of the maternity pen as a sick pen, were not associated with the magnitude of the difference in calf shedding between 2000 or 2001 and 2009.

Environmental samples

Comparable numbers of each environmental sample type were collected from each herd in seasonally matched sample dates in 2000 or 2001 and 2009 (**Table 9**). For all environmental samples, 7% (7/104) and 16% (17/108) of samples were positive for *Salmonella* in 2000 or 2001 and 2009, respectively (**Table 9**). Adjusting for the non-independence of samples within herds, the proportion of environmental samples that were positive for *Salmonella* was significantly higher in 2009 relative to 2000-2001. An environmental sample collected in 2009 had 5.27 times the odds of being positive relative to an environmental sample collected from the same set of herds in 2000 or 2001. The proportion of environmental samples positive was still significantly different between time points even while controlling for herd size (**Table 10**). Larger herds had a significantly larger proportion of environmental samples positive. This result suggests that the increases in the prevalence over time cannot be entirely explained by increases in herd size. Model fit was significantly better using only environmental samples. The chi-square residuals/degrees of freedom was 0.99, indicating adequate model fit.

Discussion

This study used a retro-prospective study design to determine changes in the prevalence of *Salmonella* between 2000-2001 and 2009. Increases in herd sizes and changes in management practices were significantly associated with within-farm differences in prevalence estimates across years.

Dairy farms harbor zoonotic *Salmonella* subtypes that are important to public health (Alcaine et al. 2006; Gupta et al. 2003); however, the distribution of serotypes between slaughter isolates and human clinical isolates is different, demonstrating that many of the serotypes of *Salmonella* shed by cattle pose less public health hazard (Sarwari et al. 2001). Dairy farms nonetheless harbor strains with high human health impact, including MDR *Salmonella* Newport and Typhimurium (Glynn et al. 1998). Additionally, ongoing diversification within this population may result in the emergence of strains with high human health impacts.

In the study from which the retrospective data were retrieved, a total of 32 Michigan dairy farms were sampled between 2000 and 2001, over half of those herds (18) agreed to participate in sampling again in 2009. The lack of availability of more herds for this study limits the statistical power and the ability to address associations with a larger number of management practice changes.

Nonetheless, changes in the prevalence of *Salmonella* in adult cow samples provided by this study are consistent with estimates provided by the NAHMS (NAHMS). In this study, 9% and 12% of adult cows were shedding *Salmonella* in 2000 or 2001 and 2009, respectively. Studies by NAHMS showed that the proportion of adult cows shedding *Salmonella* was 5.4%, 7.1% and 13.7% in 1996, 2002, and 2007, respectively (USDA 2011b).

The prevalence of *Salmonella* significantly increased with time for cow and environmental samples, but was not different for calves. The difference in findings for cow and calf samples may be in part due to a larger number of cow samples. However, using a single seasonally matched sampling visit may miss outbreaks of shorter duration that are perhaps more frequent in calves, and thus provide a less precise prevalence estimate.

Increases in *Salmonella* prevalence may be due to changes in the microbial environment, increases in the frequency of transmission, or changes in the population of *Salmonella*. Changes in dairy farm management practices may alter the frequency of transmission and/or the microbial environment for *Salmonella*. Dairy farm changes over the past ten years most notably include increases in the average herd size. In the NAHMS studies, the percentage of sampled herds with over 500 cows was 19%, 26%, and 31% for studies in 1996, 2002, and 2007, respectively (USDA 2011b), which may in part explain increases in national-level prevalence estimates. However, increases in prevalence across study years were noted within each herd size category (USDA 2011b). Larger herd sizes have been associated with a higher prevalence in adult cattle, (Habing et al. 2012; Ruzante et al. 2010; Kabagambe et al. 2000; Blau et al. 2005; Warnick et al. 2001), higher contamination of bulk tank milk (Ruzante et al. 2010), higher incidence of clinical salmonellosis (Cummings et al. 2009b), higher shedding in calves (Losinger et al. 1995), and a higher rate of introduction of MDR strains (Adhikari et al. 2009b). The association with larger herds may be due to the more frequent availability of fresh cows, sick cows, and calves (Warnick et al. 2001), differences in management practices and biosecurity between small and large dairy farms, or inherent differences in transmission dynamics for larger populations of animals (Gardner et al. 2007). Regardless, increases in herd size could plausibly be expected to result in higher prevalence of shedding on dairy farms. In this study, two of the six (33%) herds with a at

least a 200-cow increase in herd size also had a significant increase in the prevalence of cow or environmental samples. whereas two of ten herds without similar herd size increase had a significant increase in the prevalence of *Salmonella* in cow or environmental samples.

Temporal changes in the population of *Salmonella* that lead to improved fitness characteristics or ability to colonize dairy animals would likely lead to increases in prevalence. The prevalence and AMR of *Salmonella* concurrently increased and decreased between the two most recent cross-sectional studies by the NAHMS (USDA 2011b). Changes in AMR between 2000-2001 and 2009 for the set of farms included in this study has been previously described (Habing, et al. 2012). Briefly, the proportion of *Salmonella* resistant to any antimicrobial was 27% and 1% in 2001 and 2009, respectively (Habing et al. 2012). The prevalence of clonal MDR Typhimurium DT104 has also been declining within the United States, and may be displaced by other pansusceptible strains. Declines in AMR, or displacement of resistant strains by pansusceptible strains may lead to a population of *Salmonella* better adapted for asymptomatic colonization in dairy cattle, resulting in higher prevalence estimates.

The number of farms available for this study limited the ability to examine a large number of practices. Therefore, this study specifically focused on changes in management practices previously found to be significantly associated with *Salmonella* shedding in 2000-2001 Fossler et al. 2005a; Fossler et al. 2005b). This study design enabled assessments of the prevalence within the same farm at time points when management practice changes had occurred, offering an advantage over shorter-term longitudinal studies. This design also offers an assessment of the impact of potential positive interventions within herds on *Salmonella* shedding. However, the association with management practice changes and prevalence changes may be confounded by concurrent temporal changes in the population of *Salmonella* or other

management practices not assessed. This is the first study to examine the effect of managmenet practice changes on longer-term changes in prevalence; however, the management practices examined in this study have been associated with *Salmonella* shedding in other epidemiologic studies. Calves on medicated milk replacer had a lower prevalence of *Salmonella* shedding in two studies (Berge et al. 2006; Losinger et al. 1995). Also, the use of liquid manure was an identified risk factor in research utilizing data collected by NAHMS (Ruzante et al. 2010; Habing et al. 2012). This result in particular is consistent with experimental work that has shown longer persistence of *Salmonella* in manure slurry relative to static manure piles (Nicholson et al. 2005; You et al. 2006; Toth et al. 2011).

In this study, changes in prevalence were not perfectly correlated with increases in herd size or changes in management practices. The largest difference in prevalence was for a farm that had a similar herd size and no reported changes in the management practices examined. The observed increases are likely explained by combinations of *Salmonella* population shifts and unobserved dairy farm environment changes.

Conclusions

In conclusion, shedding of *Salmonella* in adult dairy cattle significantly increased between 2000-2001 and 2009. These increases are in agreement with national-level prevalence estimates, and may lead to more frequent contamination of the food supply. In this study, increases in prevalence were significantly associated with herd size increases and changes in management practices, including wider usage of liquid manure and grazing pastures where manure had been applied. However, long-term studies with larger numbers of herds and more frequent sampling points would be useful to evaluate the impact of management practices.

Table 9 - Number of samples (proportion positive) collected on eighteen Michigan Dairy Farms

			Summer-matched visits			
Sample type	**2000-2001**		**2000 or 2001**		**2009**	
Cow samples						
Healthy	2994	(0.05)	518	(0.09)	238	(0.14)
Cull	77	(0.03)	8	(0.13)	33	(0.06)
Close-up	176	(0.02)	38	(0.03)	73	(0.11)
Fresh	257	(0.06)	42	(0.12)	76	(0.12)
Sick	165	(0.04)	32	(0.13)	44	(0.23)
Total (cows)	**3669**	**(0.05)**	**638**	**(0.09)**	**464**	**(0.13)**
Calf Samples						
Calves	770	(0.06)	145	(0.08)	163	(0.07)
Environmental samples						
Sick pen	50	(0)	9	(0)	11	(0.45)
Manure storage area	103	(0.14)	18	(0.17)	17	(0.29)
Haircoat of cull cow	57	(0.07)	8	(0)	11	(0)
Maternity pen	85	(0.04)	16	(0)	18	(0.17)
Milk filter	99	(0.05)	17	(0.06)	16	(0.06)
Waterer	105	(0.06)	18	(0.11)	18	(0.06)
Calf pen	104	(0.04)	18	(0.06)	17	(0.12)
Other sample types	306	(0.03)	--		--	
Missing information	10		3		4	(0.25)
Total (environment)	**909**	**(.05)**	**104**	**(0.07)**	**108**	**(0.16)**
Total	**5358**	**(0.05)**	**890**	**(0.09)**	**739**	**(0.12)**

Table 10 – Results of a generalized linear model for changes in the prevalence between 2000-2001 and 2009 for Michigan dairy herds sampled in both time points. The effect of year unadjusted and adjusted for total herd size are shown.

Calf samples					
Variable	**Level**	**Estimate**	***p* value**	**OR**	**(95% CI)**
Year	2009	-0.37	0.44	0.69	(0.26 - 1.84)
	2000-2001	--			
Year	2009	-0.82	0.21	0.44	(0.55 - 1.85)
	2000-2001	--			
Herd size	--	0.002	0.29		
Cow samples					
Variable	**Level**	**Estimate**	***p* value**	**OR**	**(95% CI)**
Year	2009	0.77	< 0.001	2.16	(1.38 - 3.37)
	2000-2001				
Year	2009	0.01	0.98	1.01	(0.55 - 1.85)
	2000-2001	--			
Herd size	--	0.005	< 0.001		
Environmental samples					
Variable	**Level**	**Estimate**	***p* value**	**OR**	**(95% CI)**
Year	2009	1.66	0.001	3.5	(2.16 - 12.84)
	2000-2001				
Year	2009	1.25	0.02	3.38	(1.23 - 9.93)
	2000-2001				
Herd size	--	0.002	0.07		

Figure 2 – Scatter plot depicting the association between changes in *Salmonella* prevalence and change in herd size. Y axis, cow prevalence difference between 2009 and 2000 or 2001; X axis, difference in herd size (adult cow numbers) between 2009 and 2000 or 2001

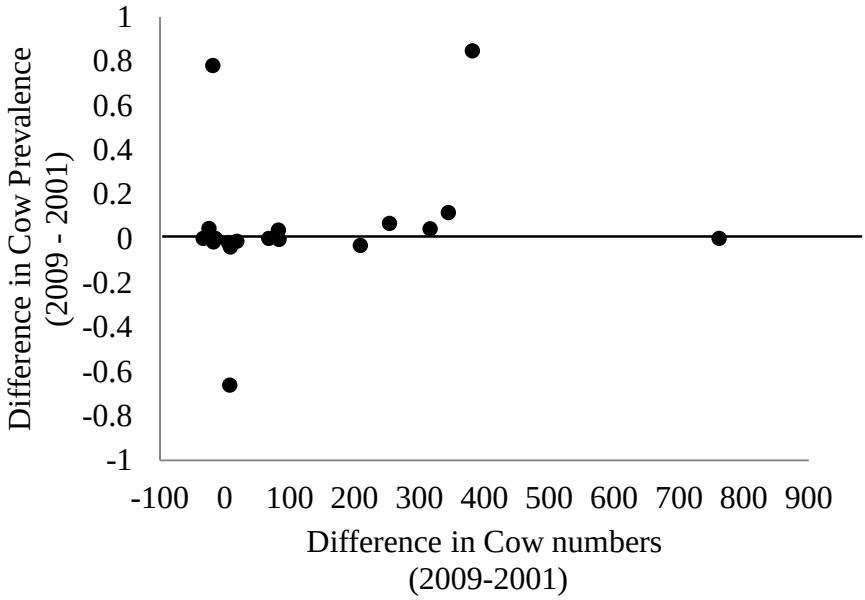


Figure 3- Scatter plot depicting the association between changes in *Salmonella* prevalence and changes in management practices between 2009 and 2000 or 2001.

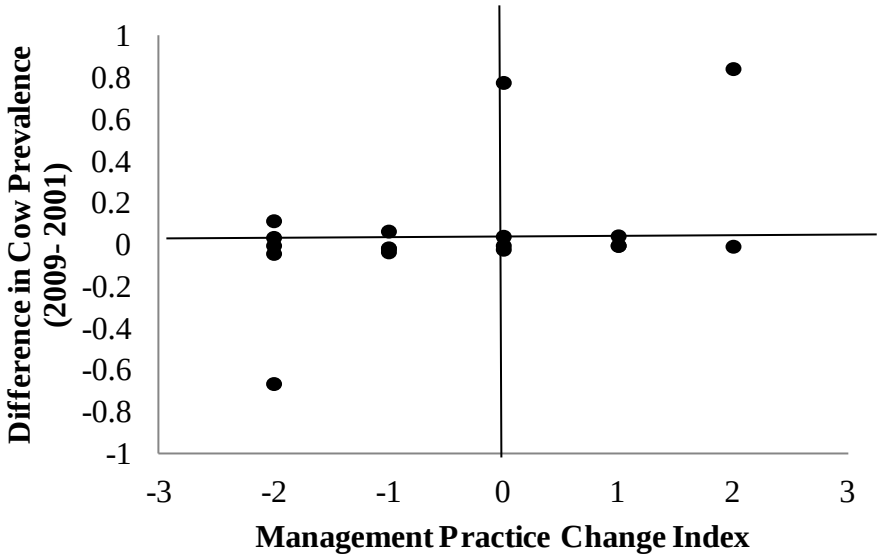


Table 11 – Changes in management practices for eighteen Michigan dairy herds. Herds with significant differences ($p < 0.05$) differences between the 2009 summer prevalence and the 2000-2001 summer prevalence for cow samples are highlighted. Green, changes in management practices expected in increase *Salmonella* shedding; Red, changes in practices expected to decrease *Salmonella* shedding

Farm	Herd size change[5]	Management Practice Index	Grazing after manure application[1]	Slurry application[2]	Feed enclosed building[3]	Rumensin in heifers[4]
109[a]	382	2	Started	Started	Both years	Neither
101[a]	-18	0	Neither	Neither	Neither	Both years
121[a]	345	-2	Stopped	Stopped	Neither	Neither
125	254	-1	Neither	Both years	Started	Neither
131	-24	1	Neither	Neither	Stopped	Neither
122	317	0	Neither	Started	Started	Both years
114	83	-2	Stopped	Both years	Started	Neither
108	68	1	Neither	Started	Started	Stopped
118	-33	-2	Neither	Both years	Started	Started
126	762	1	Started	Both years	Neither	Neither
132	-14	0	Both years	Neither	Neither	Both years
102	84	2	Started	Started	Both years	Neither
128	19	-1	Neither	Neither	Started	Neither
119	-17	-1	Neither	Both years	Neither	Started
127	6	0	Neither	Neither	Started	Stopped
129	209	-1	Neither	Both years	Started	Both years
112	9	-2	Stopped	Neither	Started	Neither
111[b]	8	-2	Both years	Both years	Started	Started

[1] Cows eating roughage from fields where manure was applied in solid or liquid form

[2] Surface application of slurry on owned or rented land

[3] Purchased concentrates and protein feeds stored in an enclosed building (All vs some or none)

[4] Use of rumensin in weaned calves or heifers

[5] Number of adult cattle in 2009 – number of adult lactating cattle in 2000 or 2001.

[a] 2009 cow or environmental prevalence significantly higher ($p<0.05$) than 2000 or 2001

[b] 2009 cow or environmental prevalence significantly lower ($p<0.05$) than the 2000 or 2001

Table 12 – Proportion of samples positive (total samples) for herds with significant increases or decreases in the within-farm prevalence between 2000 or 2001 for calf, cow, or environmental samples.

Farm	2000 or 2001		2009		p value
Calves					
129	0.53	(15)	0	(15)	0.001
Cows					
101	0.03	(30)	0.79	(24)	<.001
109	0	(40)	0.85	(26)	<.001
111	0.73	(40)	0.04	(24)	<.001
Environmental samples					
109	0	(8)	0.83	(6)	0.003
121	0	(12)	0.43	(7)	0.0361

Table 13 – Comparisons of the prevalence of *Salmonella* for the same herds in the summers of 2000 or 2001 and 2009.

		2009 Prevalence		
		0	**0-10%**	**>10%**
2000-2001 prevalence	**0**	3	2	1
	0-10%	5	1	2
	>10%	0	2	2

CHAPTER FOUR

Changes in the Distribution, Diversity, and Genetic Relatedness of *Salmonella* on Michigan Dairy Farms between 2000-2001 and 2009

Structured Abstract

Objective: Determine changes in the population of serotypes, sequence types, and pulsotypes on Michigan dairy farms between 2000-2001 and 2009**.**

Design: Retro-prospective

Sample Population: Eighteen Michigan dairy farms in 2000-2001 and 2009

Procedure: Stored *Salmonella* isolates recovered during a 2000-2001 longitudinal study on Michigan dairy farms were retrieved. Fecal and environmental samples were prospectively collected from the same 18 Michigan dairy farms in 2009 using comparable sample collection techniques. Serogroups, serotypes, multilocus sequence types, and PFGE banding patterns were identified for isolates from 6 *Salmonella*-positive farms in both 2000-2001 and 2009. Within-farm changes in the prevalence and distribution of multi-locus sequence types and pulsotypes were determined across five sampling visits between 2000-2001, and between 2000-2001 and 2009.

Results: The distribution of serogroups was significantly different between 2000-2001 and 2009; however, there was substantial overlap in the populations between the two time points. Eighty percent of the isolates recovered in 2009 were serotypes that were previously recovered from the same set of farms 10 years prior. Likewise, 16% of the 2009 isolates were pulsotypes that had been recovered 10 years prior. Serotypes recovered in both time frames most frequently had distinct PFGE patterns; however, PFGE patterns of isolates transiently recovered on two farms in

2001 were recovered from the same farm in 2009. MDR resistant subtypes of serotype Senftenberg and Typhimurium were recovered in 2000-2001, and genetically distinct, pansusceptible subtypes of the same serotypes were recovered in 2009.

Conclusions:

Serotypes and sequence types present in both time frames had high genetic relatedness. Indistinguishable pulsotypes were recovered within the same farm in 2000 or 2001 and 2009 for two farms, suggesting long-term persistence. There was substantial overlap in the population of *Salmonella* sequence types and pulsotypes; however, the distribution of sequence types was significantly different between time frames.

Introduction

Salmonella is a well-known cause of foodborne outbreaks and illnesses worldwide. Within the United States, it accounted for 23% of foodborne outbreaks, and 62% of hospitalizations resulting from foodborne illnesses (CDC 2011b). The severity of illnesses in patients with salmonellosis can range from mild transient diarrhea to life threatening septicemia requiring antimicrobial therapy. AMR in *Salmonella* limits the therapeutic options for clinicians and is associated with higher hospitalization rates, bloodstream infection rates, and higher hospital costs (Varma et al. 2005a; Martin et al. 2004). Livestock farms, including dairy farms, are important reservoirs of *Salmonella* that cause illness in humans. Cattle asymptomatically shedding *Salmonella* routinely pass through slaughter plants. The serotypes, resistance phenotypes, and genetic subtypes of *Salmonella* present on dairy farms have significant overlap with strains that cause disease in humans (Alcaine et al. 2006; Wedel et al. 2005; FDA 2010).

Prior cross-sectional studies conducted by the NAHMS show important changes in national-level estimates of AMR over time. Specifically, the proportion of *Salmonella* isolates resistant to at least one antimicrobial decreased from 18% to 8%, while the herd-level prevalence of *Salmonella* increased from 20% to 30% in studies conducted in 2002 and 2007, respectively (USDA 2011b). The relative prevalence of serotypes across these studies also changed, with increases in the prevalence of serotypes Cerro, Kentucky, and Muenster (USDA 2011b).

Consistent with the NAHMS data, a retro-prospective study by this group showed concurrent increases in the prevalence of shedding and decreases in the AMR of *Salmonella* on Michigan dairy farms between 2000-2001 and 2009 (Habing et al. 2012). Taken together, these data suggest important shifts in the population of *Salmonella* of dairy farms.

Changes in AMR of *Salmonella* on dairy farms can occur through horizontal transfer of mobile genetic elements as well as dissemination of clonal strains (Alcaine et al. 2005). In particular, the emergence and dissemination of multidrug resistant clonal subtypes, such as *Salmonella* Typhimurium DT104 and MDR *Salmonella* Newport have had important impacts of the types and frequency of AMR in *Salmonella* on dairy farms (Davis et al. 1999; Berge et al. 2004). Emergence of novel subtypes can result in temporal patterns of displacement of one *Salmonella* strain by another. For instance, chloramphenicol resistant *Salmonella* DT104 appeared to displace other strains of *Salmonella* Typhimurium within a collection of isolates from a veterinary diagnostic laboratory (Davis et al. 1999). More recently, there was also a temporal pattern for *Salmonella* Typhimurium sequence type (ST) 213 was displacing its founding genotype, ST19 (Wiesner et al. 2009). Observational research of *Salmonella* shedding on dairy farms has also shown *Salmonella* to persist for many years. Recently, persistence of *Salmonella* Cerro within a dairy herd was demonstrated for three years, followed by a gradual shift in the population to a different serotype. Clearly, longer-term longitudinal studies are needed to understand the frequency and drivers of population changes within dairy farms.

Serotype and subtype identification is necessary to understand the genotypic population shifts that may be responsible for the observed temporal changes in prevalence and AMR. Serotypes, defined by the serologic identification of the flagellar and LPS-associated antigens, remain useful classifications to define epidemiologically relevant groups that differ in host specificity, virulence, and regional distributions (Sarwari et al. 2001; Galanis et al. 2006; Heithoff, 2012). PFGE also continues to be a reliable method that possesses high capacity for differentiation of *Salmonella* strains (Zhao et al. 2003b; Foley et al. 2007). However, gene sequencing subtyping techniques provide less ambiguous data and the ability to infer

phylogenetic relationships. Previous molecular epidemiological studies of *Salmonella* often use laboratory collections of isolates, which provide the necessary diversity to study phylogenetic relatedness; however, these inferences can't be extrapolated to the population of *Salmonella* on dairy farms, or examine within-farm changes over time. Additionally, most observational studies of *Salmonella* shedding on dairy farms are cross-sectional or of limited duration, which may not be sufficient to understand long-term population changes caused by emergence or disappearance of *Salmonella* strains.

Given the observed changes in prevalence and AMR of *Salmonella* on Michigan dairy farms over the past 10 years (Habing et al. 2012), we hypothesized that the serotypes, sequence types and PFGE banding patterns of *Salmonella* serotypes recovered in 2009 are distinct from *Salmonella* collected the same farms in 2000-2001. The objectives of this study were to determine the relatedness, diversity, and distribution of *Salmonella* subtypes recovered from Michigan dairy farms over five sampling visits in 2000-2001, and the differences between the 2000-2001 and 2009 *Salmonella* population.

Materials and Methods

Study Design

Data for this retro-prospective study consists of two components: retrospective data retrieved from a study of *Salmonella* on Michigan dairy farms, and data collected prospectively 10 years later from the same Michigan dairy farms. Retrospective data were retrieved from a longitudinal study of *Salmonella* shedding on randomly selected dairy farms in Michigan, New York, Wisconsin, and Minnesota (Fossler et al. 2004). Stored *Salmonella* isolates collected from Michigan dairy farms between June 2000 and September 2001 were retrieved from the Center

for Comparative Epidemiology (CCE) at Michigan State University. Samples from the same farms were collected prospectively in July and August of 2009.

Farm Selection

In 2000-2001, 31 dairy farms in Michigan were sampled that met the following criteria: less than 100 miles from Michigan State University, milking greater than 30 Holstein cows, raising their own calves for replacements, and shipping milk year-round. For the data collected in 2009, all Michigan dairy farms that participated in 2000-2001were recruited.

Sample Collection

For the purposes of this manuscript, the word "sample" is used to refer to either animal fecal samples or environmental swabs collected from dairy farms. Comparable sampling plans for collecting fecal and environmental samples were used in both 2000-2001 and 2009. In 2000-2001, farms were sampled every other month, resulting in a minimum of five sampling events per farm. In 2009, farms were sampled once, and sampled twice if the farm was negative for *Salmonella* on the first round of sampling, and had a greater than three percent shedding prevalence in 2000-2001. Fecal samples were collected from the rectum of dairy cattle using a single use rectal sleeve, and from calves using digital rectal retrieval. In both 2000-2001 and 2009, healthy lactating cows and "target" animals were sampled from each farm. Target animals were defined as dairy animals most likely to be shedding *Salmonella*, including pre-weaned calves, cows identified as sick by the farm management, cows within 14 days of their calving date, and cows scheduled to be culled within 14 days. Target animals were preferentially sampled to increase the number of *Salmonella* isolates recovered and most accurately define the population of *Salmonella* within each farm. The number of samples collected was calculated to

provide a 95% probability of recovering at least one *Salmonella* positive sample. Similar sample size calculations for fecal and environmental samples were used in 2000 and 2009, and have been previously described (Fossler et al. 2004). Systematic sampling was used to obtain a representative sample of healthy cows and target animals. Environmental samples were taken using gauze swabs soaked with double-strength skim milk. Samples were taken from cow environments, including the maternity pen, sick pen, cull cow hide, milk filter, and manure storage area. Samples from calf environments included a composite sample from multiple calf pens. All samples were stored in commercial bags, placed in a cooler with ice, and submitted to the Microbial Epidemiology Laboratory at the Center for Comparative Epidemiology the following day.

Salmonella Isolation

Isolation of *Salmonella* was performed in the same laboratory with highly similar protocols in 2000 and 2009 (Fossler et al. 2004). With the exception of a confirmatory step (urea agar used in 2009), and the number of colonies chosen for confirmatory steps (up to five in 2009, and up to two in 2000), the protocols for the isolation and confirmation of *Salmonella* from fecal and environmental samples were identical. Samples were enriched by adding tetrathionate broth to achieve a 1:10 dilution and incubating for 48 h at 37 °C. The enriched sample was streaked onto XLT4 agar and incubated for 24 h at 37 °C. In 2009, up to five suspect colonies from XLT4 agar, (red or yellow with black centers) were inoculated onto TSI and urea agar slants, and incubated for 24 h at 37 °C. In 2000, up to two suspect colonies from XLT4 agar, (red or yellow with black centers) were inoculated onto TSI only, and incubated for 24 h at 37 °C. Colonies with test results typical for *Salmonella* (alkaline/acid/H2S positive and urease negative) were then inoculated onto lysine-iron agar and Simmons citrate agar slants. Colonies that were lysine

decarboxylase and hydrogen sulfide positive in lysine-iron agar (purple slant and purple-black butt) as well as positive in Simmons citrate (blue) were considered positive for *Salmonella*. *Salmonella* isolates harvested in 2000 were frozen in tryptic soy broth/glycerol solution at −80 °C and stored in cryovials. In 2009, these were retrieved, and underwent further biochemical confirmation before antimicrobial susceptibility testing. Isolates were stabbed onto a TSA slant, and stored at room temperature prior to antimicrobial susceptibility testing.

Antimicrobial Resistance Testing

Salmonella isolates collected in the 2000-2001 study were tested concurrently with the 2009 isolates using the same commercially prepared microbroth dilution antimicrobial panels. In 2000-2001, up to two *Salmonella* colonies per sample were chosen for testing. In 2009, up to five isolates per sample were chosen for testing. The antimicrobial resistance panel contained a prepared range of concentrations for the following 15 antimicrobials: amikacin, amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. Quality control tests were performed using E. coli ATCC 25922 for all panels, and were all within acceptable limits. Colonies identified as *Salmonella* were streaked to Mueller Hinton agar and incubated for 18–24 h at 37 °C. Testing was performed according to the instructions from the manufacturer of the automated microbroth dilution system (Trek Diagnostic Systems, Inc.), and panels were read with an autoreader. Breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) were used to classify isolates as susceptible, intermediate, or resistant (CLSI 2010). No CLSI interpretive criteria were available for ceftiofur or streptomycin, so breakpoints presented in the NARMS 2007 Annual Report were used (FDA, 2010).

Pulsed-Field Gel Electrophoresis

Up to two randomly selected isolates from each fecal or environmental sample underwent PFGE, and were included in subsequent analyses. If more than two isolates were recovered from a single sample, then two isolates were randomly chosen to undergo PFGE. PFGE was conducted at the DCPAH using a CDC standardized protocol (31). Briefly, DNA was prepared and incubated with the *XbaI* rare-cutting restriction enzyme. The resultant macrorestriction fragments were prepared within 1% agarose gel plugs and separated using pulsed field gel electrophoresis with an initial switch time of 2.2s, final switch time of 63.8s, and a total run length of 16.2 hrs. Gels were stained with 400 mL ethidium bromide solution and photographed with GelDoc Imager (BioRad). Band patterns were imported into the Bionumerics (v. 4.2 Applied Maths, Kortrijk, Belgium) and standardized against *Salmonella* Braenderup H9812. If two isolates from the same sample had indistinguishable PFGE patterns, then one isolate was included in the statistical analysis and summary statistics.

Serotype Identification

Serotype identification was performed at the DCPAH at MSU using the Kauffman-White scheme. The LPS-associated antigen (O-antigen) and the flagellar associated antigens (H-antigens) were identified using slide and tube agglutination techniques, respectively (23).Where a group of isolates collected from the same farm on the same day had indistinguishable PFGE banding patterns, only one isolate was serotyped, and the remaining isolates are reported as the same serotype.

Multilocus Sequence Typing

To define sequence types, the partial gene sequence of seven housekeeping genes (*thrA, purE, sucA, hisD, aroC, hemD,* and *dnaN*) was determined according to a standard MLST protocol for *Salmonella* (http://mlst.ucc.ie). Isolates with different PFGE patterns, collected from different farms, or collected in different time frames (2000-2001 or 2009) were selected for MLST. A 96 well plate containing 50 µL of culture was spun down and the supernatant was discarded. Cell pellets were resuspended in 50 µL of dH2O and heated to 59 C^{o} for 10 minutes to lyse the cells. The plate was centrifuged to pellet the debris and an aliquot of the crude extract was diluted 1:10 into dH2O. One microliter of this dilution was then used as template in a 10ul PCR reaction containing 20mM Tris-HCl (pH8.4), 50mM KCl, 1.5mM MgCl2, 0.2mM dNTP's, 0.4uM each primer, and 0.5U Taq DNA polymerase (Invitrogen). Primer sequences and amplification conditions were obtained from the MLST database at the ERI, University College Cork (http://mlst.ucc.ie). Following amplification approximately 20ng of PCR product was treated with ExoSAP-IT (Affymetrix/ USB products) to inactivate remaining primers and nucleoside triphosphates and then used as template in a sequencing reaction with the appropriate sequencing primer using Applied Biosystems v3.1 BigDye chemistry and run on a 3730xl Sequencer (Applied Biosystems). Chromatograms were uploaded into computer software (DNASTAR Lasergene Seqman Pro v. 8.1.5) for trimming, alignment and editing. Edited sequences were uploaded to the MLST website ((http://mlst.ucc.ie) to identify the allelic numbers and sequence types for each isolate.

Statistical Analysis

Descriptive statistics were generated for the proportion of samples positive for *Salmonella*, proportion of isolates resistant to any antimicrobial, and the frequency of sequence

types (STs) and AMR profiles. The frequency distributions of serogroups and STs were compared using a chi-square test of independence. The PFGE data were analyzed using BioNumerics (v. 4.2). Dendrograms were constructed by applying hierarchical agglomerative clustering techniques (unweighted pair group method with arithmetic means) to similarity matrices calculated using the dice coefficient of similarity. Band tolerance settings of 1.5% were used. Permutation testing was applied to the similarity matrices to test the null hypothesis that the serotypes were not distinct between groups within farms, between farms, or between years (2000-2001 or 2009) (Sickle 1997). Permutation testing was performed separately for each serotype, and utilized where a serotype was recovered from multiple samples within each comparison group. Similarity matrices were exported from Bionumerics (v. 4.2) and imported into SAS software (v. 9.1.3). Mean between-group ($\bar{B}$) and within-group ($\bar{W}$) similarity was calculated by averaging the dice coefficients where isolate pairs were in different or same comparison groups, respectively. Statistical significance of the ratio ($M_{obs} = \frac{\bar{B}}{\bar{W}}$) was tested by comparing the magnitude of M_{obs} to the same test statistic recalculated for 500 permutations of the comparison group label. The *p*-value was calculated as the proportion of permuted test statistics that are less than M_{obs}, and can be interpreted as the probability of obtaining a test statistic that is smaller than M_{obs} with a random permutation of the group labels of the isolates. *P* values less than 0.05 were considered to consistent with distinct populations of subtypes between the comparison groups.

Simpson's index of diversity (*D)* (21) was calculated within each time frame

$$D = 1 - \sum_{i=1}^{m} n_i(n_i - 1)/N(N - 1),$$

where *m* is the total number distinguishable subtypes, n_i is the number of isolates for each subtype *i*, and *N* is the total number of isolates. A t-test (25) was used to determine significant differences in diversity between time frames. The variance (S^2) of *D* and associated *t* statistic are calculated as follows,

$$S^2 = 4[\sum_{i=1}^{j}(p^3 - (p_i^2)^2)]/N$$

$$t = (|D_1 - D_2|)/\sqrt{S_1^2 + S_2^2},$$

where p_i is the proportion n_i/N.

Results

Recovery of *Salmonella*

Animal and environmental samples were collected from 18 Michigan dairy farms in 2000-2001 and 2009. In 2000-2001, two herds were initially visited weekly for eight consecutive visits, followed by five consecutive bimonthly visits. Sixteen herds in 2000-2001 were visited bimonthly for 5 consecutive visits. In the summer of 2009, 15 herds were visited once, and 3 herds were visited twice. In 2009, 12% (97/830) of the samples, and 10 of the 18 farms were positive for at least one isolate of *Salmonella*. In 2000-2001, 14 of the same 18 farms and 6% (264/5,358) of samples were positive. Detailed descriptions of the within-farm changes in prevalence for all 18 herds are provided elsewhere (Chapter 2). Serotypes, sequence types, and PFGE patterns were determined for isolates from farms where at least two isolates were recovered in both sampling time frames. Eight farms were positive in both time points, but two farms had only one isolate in either time point, leaving a total of six farms. Excluding isolates

from the same sample with indistinguishable PFGE patterns, a total of 273 and 77 isolates were recovered on these six farms in 2000-2001 and 2009, respectively. The remainder of this analysis focuses on the genetic relatedness, diversity, and distribution of the serotypes and pulsotypes of *Salmonella* from these six farms in 2000-2001 and 2009.

Salmonella serotypes

Thirteen different serotypes were recovered in 2000-2001, and ten different serotypes were recovered in 2009 (**Table 16**). A total of six serotypes (**Table 18**) were recovered in both 2000-2001 and 2009. Of these six serotypes, three were recovered from the same farm at both time points, and three were recovered from different farms.

Multilocus Sequence Types

A total of 11 and 9 different sequence types (STs) were recovered in 2000-2001 and 2009, respectively (**Table 16**). In all but one case, there was a single ST for each serotype; regardless of the farm or year the sample was taken. A novel ST of serotype Hartford (recovered in 2000) differed by a single base pair in the *dnaN* allele from ST 405 of serotype Hartford recovered from in 2009. Two serotypes, Typhimurium and 4:5:12:i:-, known to be genetically similar, were both ST 19 (Guerra et al. 2000).

Antimicrobial Resistance in 2000-2001 and 2009

The proportion of *Salmonella* resistant to any antimicrobial was higher in 2000-2001 than in 2009. Detailed analysis on within-farm changes in the AMR of *Salmonella* are available elsewhere (Habing et al. 2012). Briefly, the frequency of AMR was lower in 2009 relative to 2000-2001. Changes in AMR were a result of with a higher frequency of MDR subtypes in 2000-2001, as well as decreases in the MICs of isolates classified as susceptible. Excluding isolates

from the same sample with indistinguishable PFGE patterns, the proportion of isolates resistant to at least one antimicrobial was 16 percent (43/271) and 1.3 percent (1/77) in 2000-2001 and 2009, respectively. The proportion of isolates resistant varied by season (**Table 21**), but for the summers of 2000, 2001 and 2009, the proportion of isolates resistant was 84%, 27%, and 1%, respectively (**Table 21**). Across all years, resistance to any antimicrobial was detected only in serotypes Typhimurium, Senftenberg, and Bovis-Morbificans (**Table 18**). MDR subtypes of Typhimurium (ACSSuT) and Senftenberg (GKSSuT) were recovered in 2000-2001 from farms 101 and 129, respectively (**Table 18**). In 2009, strains of Typhimurium and Senftenberg (farms 114 and 121, respectively) were susceptible to all antimicrobials. Subtypes of Bovis-Morbificans recovered in 2000 were predominantly susceptible to all antimicrobials in both 2000-2001 and 2009; however, single isolates resistant to streptomycin and sulfisoxazole were recovered in 2000-2001, and a ceftriaxone resistant strain of Bovis-Morbificans was recovered from the same farm in 2009. Other serotypes recovered in both years were susceptible to all tested antimicrobials.

Pulsotypes 2000-2001

Thirty-eight distinguishable pulsotypes were recovered over the 2000-2001 sampling period. The number of pulsotypes from each positive herd ranged from 1 to 13 (**Table 16**). Eleven pulsotypes persisted within the 2000-2001 sampling period, and were recovered on multiple sampling visits from the same farm. The number of days between the first and last recovery of a subtype ranged between 21 and 290 days (**Table 19**). Three pulsotypes (BM2, BM3, and Muens1) on farms 111 or 114 persisted for the duration of the 2000-2001 study (290 days), and were recovered on all sampling visits.

Two herds accounted for 73% of the positive samples during the 2000-2001 time frame. Persistent, high prevalence herd infections on farms 111 and 114 were primarily caused by a single dominant pulsotype. On farm 111, 10 pulsotypes and 3 serotypes were recovered (**Table 17**), but one pulsotype of Bovis-Morbificans represented 85% of the recovered isolates over the year of sampling (**Table 20**). Similarly, on farm 114, 12 pulsotypes and 3 serotypes were recovered, but a single pulsotype of Muenster represented 78% of the recovered *Salmonella* isolates over the five visits. All strains of Meunster on farm 114, including the dominant pulsotype, were susceptible to all antimicrobials at all of the 2000-2001 visits. All but two isolates of the dominant pulsotype of Bovis-Morbificans on farm 111 were susceptible to all antimicrobials.

Pulsotypes - 2009

In 2009, 10 different serotypes and 25 unique PFGE patterns were recovered from six herds (**Table 16**). Three herds had a >20% prevalence in 2009, and were infected with serotypes Montevideo, Typhimurium and Bovis-Morbificans (**Table 17**). A single pulsotype accounted for 50%, 48%, and 69% of the positive samples within each farm, respectively.

Changes in the distribution of serogroups and sequence types between 2000-2001 and 2009

The overall distribution of serogroups and sequence types present on farms in 2009 was significantly different ($p<0.05$) than the distribution present in 2000-2001 (**Table 14**). Serogroup C2 and E1 comprised 46% and 35%, respectively, of the recovered isolates in 2000-2001. In 2009, however, only 13% of the isolates were serogroups C2, and serogroup E1 was not recovered. Serogroup C1 was the predominant serogroups on three of the six farms, and comprised over half of the isolates in 2009 (**Table 15**). Isolates of this serogroup, however, were

only transiently recovered in the 2000-2001 study. Although the distribution of serogroups was significantly different between years, 80% (59/77) and 16% (12/77) of the *Salmonella* isolates recovered in 2009 were serotypes and pulsotypes, respectively that had been recovered from the same 6 farms 10 years prior.

Between-year Similarity of Pulsotypes

Six serotypes were recovered in both 2000-2001 and 2009. For three farms, the same serotype/ST was recovered within the same farm in both time frames. For two of these farms, the serotypes had indistinguishable PFGE patterns in both years (**Figure 7**). In 2001, pulsotypes of Bovis-Morbificans and Senftenberg (BM3 and Senft1) were recovered from single samples on farms 125 and 121 (**Table 20**), respectively. On the same farms in 2009, the same pulsotypes were recovered from 6% (3/50) and 20% (9/46) of samples on farms 125 and 121, respectively. A single sample in 2000-2001 was positive for serotype Montevideo on farm 101, and a distinct pulsotype of serotype Montevideo was recovered from 63% of samples from the same herd in 2001. Serotypes Typhimurium, Hartford, and Mbandaka, recovered from different farms in both time frames (**Table 17**). Between years, strains had distinct but highly similar banding patterns (cophenetic similarity > 0.90) (**Table 25**). The largest distinction between years within serotypes was between MDR and pansusceptible strains. Banding patterns for MDR (2009) and pansusceptible (2001) strains of Bovis-morbificans were approximately 60% similar. MDR strains of Typhimurium recovered in 2000 were 56% similar, and significantly distinct, based on permutation testing. ($p < 0.001$) (**Figure 5**).

Between-Farm Relatedness and Distribution of Pulsotypes

Genetically distinct subtypes of the same serotype and ST were isolated from multiple farms within the same time frame. Serotypes Meleagridis, Senftenberg, Mbandaka, and Muenster were recovered from the multiple farms within the same year, and clustered separately for different farms (**Figure 8**). Permutation testing was consistent with genetically distinct groups between farms ($p < 0.05$) (**Table 23**). Other serotypes, however, were indistinguishable between farms. Two indistinguishable pulsotypes (BM3, Muenster1) were each recovered on farms 111 and 114 in the 2000-2001 sampling time frame. On farm 111, the BM3 pulsotype was recovered from over 25% of samples on each of five visits, while the Muens1 pulsotype was recovered on one visit from a single sample (**Table 20**). On farm 114, however, the Muens1 pulsotype was recovered from over 11% of samples at each of five visits, and the BM3 pulsotype was recovered only once from a single sample (**Table 20**).

Within-Farm Relatedness and Distribution of Pulsotypes

Within farms, the variability in PFGE patterns was not associated with the source of the sample. On each farm where *Salmonella* was recovered, isolates of the same serotype recovered from different areas of the farm were most frequently indistinguishable, and permutation testing was not consistent with the distinct groups of isolates ($p > 0.05$).

The distribution of pulsotypes across groups within farms was different depending on the AMR phenotype. Indistinguishable pulsotypes of MDR Typhimurium and MDR Senftenberg were recovered in 2000-2001 from cows and calves on farms 101 and 129, respectively. Significantly larger proportions of calf samples relative to cow samples were positive for any of the MDR pulsotypes within each farm (**Table 22**). By contrast, pansusceptible strains of

Typhimurium and Senftenberg were more evenly distributed between cow and calf samples (**Table 22**). On high prevalence herds in 2000-2001 (farms 111, 114) the proportion of samples positive between cows and calves was not significantly different, and showed similar seasonal variations (**Figure 4**). These results suggest that the distributional differences between cows and calves are associated with the MDR phenotype rather than a property of the specific serotype.

Variations in the prevalence of pulsotypes across sampling visits within 2000-2001 were partly explained by season. Seasonal trends were most apparent within farm 111, where the proportion of samples positive for the dominant pulsotype of Bovis-Morbificans was closely correlated between cows and calves. Seasonal trends of the dominant pulsotype within farm 114 were less apparent (**Figure 4**).

Diversity

There was a higher level of diversity of *Salmonella* in the summer of 2009 relative to *Salmonella* recovered from the same farms in the summers of 2000-2001 (**Table 21**). Simpson's index of diversity was calculated using the serotype and PFGE data for each season of 2000-2001 and 2009. Only one unique isolate from each sample was included in the calculation between each time point. Diversity was significantly higher in 2009 (0.93) relative to 2000-2001 (0.860) ($p < 0.001$). The median number of unique PFGE patterns recovered at each sampling visit was 3 in 2009 and 2 in 2000-2001. There appeared to be a seasonal component to the variability in the estimate for diversity in 2000-2001, with higher estimates in the summers of 2000 and 2001 relative to the fall and winter.

The diversity estimates were also quite variable depending on the number of farms that were included in the calculation of the estimate. Therefore, the within-farm Simpson's index of diversity was calculated for each time point. For five of the six farms were positive for *Salmonella* in both time points, the within-farm estimate for diversity was higher in 2009 relative to 2000 or 2001, but was similar between cow and calf samples. The median within-farm estimate for cow and calf samples was 0.5 and 0.45, respectively.

Discussion

This study utilizes a unique approach that enables long-term temporal comparisons of the diversity, genetic relatedness and AMR of *Salmonella* serotypes recovered from the same Michigan dairy farms. The emergence or disappearance of novel strains can cause temporal shifts in the distribution of serotypes. Likewise, changes in dairy farm characteristics or management practices over time may influence dissemination or the ability of strains to compete in their environment. Given the ability of *Salmonella* to persist within farms for long periods of time, longitudinal studies with greater temporal separation may be advantageous. This study demonstrates shifts in the population of *Salmonella* between years that influence prevalence estimates for AMR, as well as long-term persistence of pansusceptible strains of *Salmonella* within farms. Increasing prevalence and decreasing AMR over time observed in this set of farms are consistent with national-level data from the USDA (USDA 2011b). The proportion of cows and farms positive for *Salmonella* roughly doubled between 1996 and 2007, while the prevalence of AMR decreased between 2002 and 2007.

Important limitations with this research include the small number of herds that were positive for *Salmonella* in both time frames and the less extensive sampling conducted in 2009

relative to 2000-2001. Sampling in 2009 was primarily conducted in summer, while sampling in 2000-2001 was conducted in all four seasons. Estimates of diversity and AMR, where samples were collected in summer (July, August, and September) are shown in **Table 21**.

MLST has become more commonplace for subtyping foodborne pathogens, particularly *Salmonella*. MLST provides unambiguous data and valid phylogenetic inferences. In this study, however, MLST did not provide the ability to discern phylogenetic relatedness within serotypes across years. The degree of strain differentiation is in part determined by the selection of the loci and the number of loci sequenced. Most frequently, housekeeping loci are used because they are present in all non-typhoidal *Salmonella*, and are not subject to selective pressures that can result in rapid gene sequence changes (Foley et al. 2006). Studies using virulence or pathogenicity loci rather than housekeeping genes have found the levels of discrimination to be similar to PFGE (Foley et al., 2006b; Kotetishvili et al., 2002). However, most studies have found a discriminatory capacity for MLST in *Salmonella* to be less than PFGE (Sukhnanand et al. 2005; Torpdahl et al. 2005; Litrup et al. 2010; Soyer et al. 2010). Future studies utilizing additional loci with higher variability will be necessary to determine if serotypes present in both time points represent the same or distinct phylogenetic lineages.

This study provides evidence of within-farm persistence of pulsotypes for 10 years. Pulsotypes that contributed 12% of the total *Salmonella* burden on six dairy farms in 2009 were only transiently isolated from the same farms in 2000-2001. Long-term persistence within farms has been documented for serotypes Cerro, Typhimurium, and Newport (Vanselow et al. 2007; Warnick et al. 2003; Cobbold, Rice, et al. 2006) and within dairy cattle animals for up to a year (Cummings et al. 2009a). At the farm-level, long-term persistence may be caused more by environmental persistence and temporary chain infections, rather than extended excretions from

individual animals (Cobbold et al. 2006). In one study, the duration of shedding for isolates causing clinical disease did not differ by serotype (Cummings et al. 2009a); however, *Salmonella* that instead cause asymptomatic colonization of a large percentage of animals on the farm may be more apt to persist for longer periods of time.

The population of *Salmonella* serogroups in 2009 was different from the *Salmonella* population in 2000-2001. Three of the farms shifted to a C1 serogroup, and serotypes of serogroups C1 that were transiently isolated in 2000-2001 (**Table 17**), were more prevalent in 2009. Three of the four serogroups C1 serotypes were again recovered in 2009. This is in contrast to serogroups E1 and E4, which were common in 2000-2001, but rare and absent in 2009, respectively. These results show a shift in the population of *Salmonella* that was distributed across a majority of farms.

Population changes may be a result of introduction of new strains into the farm environment, within-farm diversification of the strain resulting in higher fitness, or changes in the microbial environment that result in differential fitness advantages between *Salmonella* strains. A recent study showed in greater temporal detail a within-farm serotype shift that occurred gradually over two years (Van Kessel et al. 2012). Introduction of novel strains is not an uncommon event. The rate of introduction of MDR strains (0.9/herd-year), and was significantly associated with off-farm rearing of heifers (Adhikari et al. 2009a; Adhikari et al. 2009b).

Furthermore, changes in the size and management practices of dairy farms may results in changes in *Salmonella* populations. The increase in prevalence noted in this study is in agreement with national-level U.S. estimates of *Salmonella* shedding in adult dairy cattle, which roughly doubled between cross-sectional studies in 1996, 2002, and 2007 (USDA 2011b).

Increases in dairy farm herd sizes may in particular result in higher levels of shedding (Ruzante et al. 2010; Blau et al. 2005; Cummings et al. 2009b). Changes in herd sizes and/or management practices may result in selective advantages for strains of the C1 serogroup relative to the E1 serogroup. The change in the population of serogroups noted in this study is also in agreement with changes noted in consecutive cross-sectional studies by the USDA. In the Dairy 2002 study, group E1 serotypes comprised approximately 30% of the total number of *Salmonella* isolates recovered, compared to only 15% in 2007 (USDA 2011b). The percentage of farms where serogroup E1 was recovered was 19% and 15% in 2002 and 2007 USDA Dairy studies, respectively (USDA 2011b). For *Salmonella*, considerable heterogeneity exists within serogroups (CDC 2012c); however, the O antigen may define common and ecologically important characteristics. For instance, elimination of *Salmonella* Gallinarum and *Salmonella* Pullorum from U.S. poultry flocks coincided with the rise of *Salmonella* Enteritidis, which was the same serogroup. Previous authors have suggested that the shared cell wall characteristics, allowed *Salmonella* Enteritidis to "fill the ecological niche vacated by eradication of avian pathogens" (Bäumler et al. 2000).

This study provides evidence for long-term persistence of *Salmonella* within dairy farms. Pulsotypes of Senftenberg and Bovis-Morbificans transiently recovered in 2000-2001 were recovered within same farms 10 years later at a higher prevalence (**Figure 7**, **Table 20**). Serotype Montevideo was recovered from the same farm in both time frames, but the two strains were genetically distinct (**Figure 7**). The differing PFGE profiles for serotype Montevideo may have resulted from ongoing genetic divergence within farm 101 over time, or the farm may have acquired a different subtype between sampling points. Ongoing diversification of *Salmonella* serotypes within farms has been previously reported, with associated changes in AMR (Soyer et

al. 2010; Hoelzer et al. 2010). The recovery of the same PFGE pattern in 2000 and 2009 may represent reacquisition of the same strain during the 10-year period; however, the reported high diversity of PFGE patterns within animal and human isolates of Senftenberg lends support to the conclusion that indistinguishable PFGE patterns are a result of long-term persistence rather than chance reacquisition of the same strain (Stepan et al. 2011). *Salmonella* Senftenberg was reported to persist for more than two years within a poultry farm (Pedersen et al. 2008). Persistence of *Salmonella* within dairy animals and the environment has also been previously been demonstrated (Soyer et al. 2010). *Salmonella* Typhimurium was intermittently excreted in the milk from a single animal for 2.5 years (Giles et al. 1989). Fecal excretion of *Salmonella* Newport was demonstrated for 190 days by a single animal (R. Cobbold, D. Rice, et al. 2006). Transient isolation of a pulsotype followed by reappearance years later is in agreement with a recent study where investigators showed a gradual serotype shift that occurred over a two year period (J. A. S. Van Kessel et al. 2012).

Persistence and lack of persistence of the susceptible and MDR strains between 2000-2001 and 2009 suggest that the pansusceptible strains may be better adapted for long-term asymptomatic colonization of cattle. Although AMR genes confer an advantage when selective antimicrobial pressure is present, they often result in overall decreases in the competitive fitness of the associated strain (Zhang et al. 2006). The differing ability of *Salmonella* subtypes to persist within farms over time may be characteristic of strains or of farm environments that are more or less conducive to strain survival and replication. The dominant pulsotypes (BM2 and Senft3) causing a high prevalence on farms 111 and 114, respectively (**Table 20**), were both recovered from single samples on herds 114, and 111 respectively. High and low prevalence on herds infected with the same pulsotype may be a result of environmental differences between

farms, rather than characteristic of certain strains. However, indistinguishable PFGE patterns do not rule out genetic differences that may confer advantageous fitness traits (Davis et al. 2003).

MDR strains of *Salmonella* Senftenberg and Typhimurium were recovered in 2000-2001 from farms 129 and 101, respectively. In 2009, only pansusceptible subtypes of Senftenberg and Typhimurium were recovered. Despite the differing AMR profiles over time, *Salmonella* Senftenberg was the same ST and had similar PFGE patterns (**Figure 6**) between 2000 and 2009. Strains of Typhimurium recovered in 2000 and 2009 had the same ST (19), but distinct AMR profiles and PFGE banding patterns (**Figure 5**). These results are in agreement with prior research that showed susceptible and resistant strains of Typhimurium to be the same ST. Previous authors using MLST and more diverse collections of *Salmonella* Typhimurium, showed Typhimurium to be a monophyletic serotype, with diversification predominantly a result of mutations. This is in contrast to MDR *Salmonella* Newport, where MDR strains primarily belong to one of three phylogenetic lineages (Sangal et al. 2010). The differing PFGE patterns for Typhimurium likely represent clonal replacement of DT104 with susceptible strains of Typhimurium, rather than ongoing diversification of the same strain.

The resistance phenotype, ST, and PFGE banding pattern of the MDR Typhimurium strain recovered in 2000 from farm 101 are indistinguishable from the globally disseminated *Salmonella* Typhimurium DT104 strain strain (Lan et al. 2009). *Salmonella* Typhimurium DT104 emerged as an important cause of illness in cattle and humans in the 1980's (M. K. Glynn et al. 1998; John Threlfall 2000). After the frequency of illnesses caused by DT104 peaked in the 2000's, the strain is becoming less prevalent (USDA 2008; USDA 2011b). Threlfall et al., 2006, showed that a decline in the AMR of *Salmonella* Typhimurium recovered from clinically ill patients in the UK was caused by a concurrent decline in the proportion of *Salmonella*

Typhimurium infections that were DT104. The emergence of DT104 in the 1990's was associated with the displacement of chloramphenicol susceptible *Salmonella* Typhimurium strains from a diagnostic laboratory (Davis et al., 1999), and other phage types of Typhimurium recovered from cattle in the Netherlands (Duijkeren et al. 2002; Rabsch et al. 2001). Temporal patterns of emergence and replacement of Typhimurium phage types have been previously noted (Butaye et al. 2006). In this study, recovery of pansusceptible subtypes of Typhimurium and Senftenberg in 2009 may represent clonal displacement of MDR strains recovered in 2000-2001.

Hierarchical clustering techniques (e.g. UPGMA dendrograms) are commonly applied to PFGE and other multivariate genetic data to describe the genetic relatedness and potential associations of clusters with a host, geographic location, or other variables of interest. These techniques alone, however, do not provide a formal statistical test to estimate the probability an apparent association is due to chance. Permutation testing is a multivariate statistical technique that was useful in this study to formally test the hypothesis that isolates recovered from different sources were significantly distinct, based on the PFGE banding patterns, by comparing the actual ratio of between group/within group similarity to a distribution of ratios generated through random permutations of the variable of interest. This technique was limited to situations where greater than one serotype was present in both comparison groups of interest.

The epidemiological characteristics of *Salmonella* within herds differed across herds and serotypes. Two high-prevalence herds contained primarily single, dominant pansusceptible pulsotypes that persisted for the duration of the 2000-2001 study, exhibited seasonal variation in prevalence, and were evenly distributed between adult lactating cows and preweaned calves. By contrast, herd infections caused by MDR strains of Typhimurium and Senftenberg were recovered over shorter time frame and were distributed primarily among preweaned dairy calves.

Higher frequencies of AMR in *Salmonella* in calves relative to cows has previously been reported (Cummings et al. 2009b; Berge et al. 2006; Ray et al. 2007). Increased ability for MDR strains to colonize or cause infections in calves may be related to host susceptibility or the differences in the gut environment. For example, fitness advantages of resistant E. coli in pre-weaned calves were associated with diet (Khachatryan et al. 2006). In this study, PFGE patterns of MDR strains were not different between groups within farms. Rather, MDR strains were distributed more commonly among preweaned calves than adult cows.

PFGE patterns of serotypes present on the multiple farms within the same year were most commonly significantly distinct ($p < 0.05$) (**Table 23**). The exception to this, however, was a clonal strain of Bovis-Morbificans, which was distributed across three different farms in 2000-2001, suggesting dissemination of the clonal subtype between farms. The significant variability between farms is consistent with previous research that showed herds were infected by either a single pulsotypes, or multiple pulsotypes that differed by <3 bands (Soyer et al. 2010). Farm-specific PFGE patterns may be a result of strains that are better adapted or more competitive in certain farm environments. Alternatively, limited dispersal of strains and ongoing diversification within farms over time may result in genetically distinct strains between geographic locations (Martiny et al. 2006).

There were also differences in the distribution of indistinguishable pulsotypes across farms. Indistinguishable pulsotypes did not consistently cause high-prevalence infections across herds. The dominant pulsotypes (BM2 and Senft3) causing a high prevalence on farms 111 and 114, respectively, were both recovered from single samples on herds 114, and 111 respectively. High and low prevalence on herds infected with the same pulsotype may be a result of environmental differences between farms, rather than genotypic characteristic of certain strains.

However, indistinguishable PFGE patterns do not rule out genetic differences that may confer advantageous fitness traits (Barrett et al. 2006; Davis et al. 2003). Ongoing diversification within farms may lead to evolution of strains capable of supplanting the existing *Salmonella* serotype. In one study, transient detection of a serotype was followed by gradually supplanting an existing serotype years after its initial detection (Van Kessel et al. 2012).

Based on serotyping, MLST and PFGE, the relatedness of serotypes between 2000-2001 and 2009 was high. Serotypes recovered in both time points were the same ST, with the exception of serotype Hartford. PFGE patterns were highly similar to the same serotype recovered from the same groups of farms in 2000-2001, with the exception of the banding pattern similarity between the MDR subtype of *Salmonella* Typhimurim recovered in the year 2000, and the susceptible subtype of *Salmonella* Typhimurium recovered in 2009 (**Figure 5**). As previously mentioned, large differences in the PFGE profiles for *Salmonella* Typhimurium may be an example of displacement of MDR strains by susceptible strains of the same serotype. Indistinguishable strains of Bovis Morbificans were recovered in 2000-2001 and 2009, and a single isolates of this serotype was resistant to ceftriaxone in 2009. Acquisition of ceftriaxone resistance within a single PFGE-clade has been reported (Davis et al. 2007). For other serotypes present in both years, the small differences in PFGE profiles between 2000-2001 and 2009 may be a result of diversification over time.

The genetic diversity, calculated using Simpson's index of diversity, was higher for the summer of 2009 than for summers of 2000-2001. Simpson's index of diversity represents the probability of selecting two different strains when the population is randomly sampled (Hunter &Gaston 1988). Across sampling visits in 2000-2001, the diversity exhibited a seasonal pattern, where there was a lower diversity for *Salmonella* isolates recovered in winter and spring relative

to summer sampling visits (**Table 21**). MDR strains of *Salmonella* have been previously shown to be highly clonal, with relatively low diversity (Brichta-Harhay et al. 2011). Similar protocols for environmental sampling, animal fecal sample collection, and *Salmonella* isolation were followed in both time frames. Nonetheless, differences in diversity may be due to known or unknown differences in the sampling protocols between 2000 and 2009. Known differences in sampling protocols include the number of sampling visits, number of isolates selected for confirmatory testing, and the number of samples collected at each sampling visit. The median number of samples per visit was similar in 2000-2001 and 2009 (50 and 43, respectively). Up to two and five *Salmonella* isolates were selected for confirmatory testing in 2000-2001 and 2009, respectively; however, only two randomly selected isolates were submitted for PFGE in 2009. There were five consecutive bimonthly sampling visits for each farm in 2000-2001, however; only one or two sampling visits were made within each season. The concurrent increase in diversity, decline in AMR, and increase in sample-level prevalence between summers of each time period suggests that MDR subtypes have been displaced by a more complex bacterial community.

Conclusions

This study provides insights into changes in the distribution, diversity, genetic relatedness, and AMR of *Salmonella* serotypes on Michigan dairy farms over time. The relative prevalence of serogroup C1 increased between 2000-2001, and the relative prevalence of serogroup E1 decreased. The decrease in the prevalence of AMR within this subset of farms was a result of the recovery of MDR subtypes in 2000-2001, which were subsequently not recovered in 2009. Instead, genetically distinct and pansusceptible serotypes appeared to displace the MDR subtypes within farms, and pansusceptible subtypes of the same serotype were recovered from

different farms. Two pansusceptible subtypes appeared to persist within farms between 2000 and 2009, and other pansusceptible serotypes were highly related between 2000 and 2009. The net effect of the displacement of MDR subtypes and persistence of susceptible subtypes is a shift in the population towards susceptible *Salmonella,* and a decline in the prevalence estimate for AMR. These data support the conclusion that the 2009 population of *Salmonella* on dairy farms was more prevalent, more diverse, and less AMR than the population of *Salmonella* recovered from the same subset of farms in 2000-2001. These population changes may have important implications for public health. Further understanding of the drivers of population changes may lead to positive interventions for reducing the prevalence and AMR of *Salmonella* on dairy farms.

Table 14 – Number (percentage of total) *Salmonella* isolates recovered for each serogroup on Michigan dairy farms sampled in 2000-2001 and 2009

Serogroup	**2000-2001**		**2009**	
B	20	(7)	16	(21)
C1	7	(3)	46	(60)
C2	126	(46)	10	(13)
E1	95	(35)	0	(0)
E4	23	(8)	3	(4)
K	0	(0)	2	(3)
	271	(100)	77	(100)

Table 15 – Predominant serogroup on Michigan dairy farms sampled in 2000-2001 and 2009. Indicated serogroup represents >70% of the recovered *Salmonella* on the farm in each year.

Farm	**2000-2001**	**2009**
101	B	C1
111	C2	C1
114	E1	B
121	E4	C1
125	E1	C2
129	E4	K

Table 16 – Serotypes, multilocus sequence types (STs), and number of pulsed field gel electrophoresis (PFGE) patterns of *Salmonella* recovered from the same six Michigan dairy farms in 2000-2001 and 2009

	2000-2001			2009		
Serotype	**ST**	**No. isolates**	**No. unique PFGE patterns**	**ST**	**No. isolates**	**No. unique PFGE patterns**
Serogroup B						
4,5,12:i:-				19	8	2
Brandenburg	65	3	2			
Typhimurium	19	17	6	19	8	1
Serogroup C1						
Braenderup				22	4	3
Hartford	*new	1	1	405	1	1
Mbandaka	413	1	1	413	8	4
Montevideo	138	1	1	138	29	9
Oranienburg	23	4	2			
Thompson				26	4	1
Serogroup C2						
Bovis-Morbificans	150	125	7	150	10	2
Newport	350	1	1			
Serogroup E1						
Anatum	64	2	2			
Give	654	3	2			
Meleagridis	nd	2	2			
Muenster	321	88	6			
Serogroup E4						
Senftenberg	14	23	5	14	3	1
Serogroup K						
Cerro				367	2	1
Total		271			77	

Table 17 – Number of *Salmonella* isolates of each serotype recovered from Michigan dairy farms in 2000-2001 and 2009.

	2000-2001		2009	
	Serotype	**No. isolates**	**Serotype**	**No. isolates**
Farm 101	Typhimurium	17	Montevideo	29
	Oranienburg	4		
	Hartford	1		
	Montevideo	1		
	Newport	1		
Farm 111	Bovis-Morbificans	122	Thompson	4
	Anatum	2	Hartford	1
	Muenster	1		
Farm 114	Muenster	73	Typhimurium	8
	Brandenburg	3	4,5,12:I:-	8
	Give	3		
	Bovis-Morbificans	2		
	Meleagridis	1		
Farm 121	Senftenberg	1	Braenderup	4
			Mbandaka	5
			Senftenberg	3
Farm 125	Muenster	14	Bovis-Morbificans	10
	Bovis-Morbificans	1	Mbandaka	3
Farm 129	Senftenberg	22	Cerro	2
	Mbandaka	1		
	Meleagridis	1		
Total		271		77

Table 18 – Frequency of antimicrobial resistance profiles of *Salmonella* serotypes that were recovered in both 2000-2001 and 2009.

Serotype	**2000-2001**		**2009**	
	Profile	**No. isolates**	**Profile**	**No. isolates**
Bovis-Morbificans	Susceptible	123	Susceptible	9
	S	1	CxT	1
	Su	1		
Hartford	Susceptible	1	Susceptible	1
Montevideo	Susceptible	1	Susceptible	29
Mbandaka	Susceptible	1	Susceptible	8
Typhimurium	ACSSuT	15	Susceptible	8
	ACSSu	1		
	ACSuT	1		
Senftenberg	Susceptible	1	Susceptible	3
	GKSSuT	13		
	GSSuT	4		
	GSuT	2		
	SuT	2		
	GKSuT	1		
Total		168		59

A, ampicillin; C, chloramphenicol; Cx, ceftriaxone; G, gentamicin; K, kanamycin; S, streptomycin; Su, sulfisoxazole; T, tetracycline

Table 19 – Number of days between the first and last recovery for subtypes recovered on multiple sampling visits during the 2000-2001 sampling period

Farm	Serotype	Pulsotype[1]	First recovery	Last recovery	Days between
111	BM	BM2	10/23/2000	8/9/2001	290
111	BM	BM3	10/23/2000	8/9/2001	290
114	Muenster	Muens1	11/3/2000	8/6/2001	276
114	Muenster	Muens5	11/3/2000	6/4/2001	213
111	BM	BM7	3/13/2001	8/9/2001	149
111	BM	BM5	12/20/2000	3/13/2001	83
101	Oranienburg	Orani2	8/10/2000	10/2/2000	53
114	Muenster	Muens4	4/13/2001	6/4/2001	52
129	Senftenberg	Senft3	7/31/2001	9/13/2001	44
101	Typhimurium	Typhi7	7/6/2000	8/3/2000	28
101	Typhimurium	Typhi6	6/29/2000	7/20/2000	21

[1]Pulsotypes were named according to the serotype/pulsotype combination, e.g. the first pulsotype of serotype Muenster was named Muens1, and the second pulsotype of Bovis-Morbificans was named BM2. PFGE patterns were considered distinguishable if there was at least a one band difference.

Table 20 – Proportion of samples positive for subtypes found on multiple farms in the 2000-2001 sampling period

Farm	Season/Year	Total samples	Pulsotype					
			BM2		BM3		Muens1	
Seasons, 2000-2001								
111	Fall '00	102	5	(0.05)	49	(0.48)	1	(0.01)
	Winter '01	57	0		15	(0.26)	0	
	Spring '01	48	0		14	(0.29)	0	
	Summer '01	56	2	(0.01)	29	(0.21)	0	
114	Fall '00	49	1	(0.02)	1	(0.02)	24	(0.49)
	Winter '01	52	0		0		6	(0.12)
	Spring '01	109	0		0		24	(0.22)
	Summer '01	49	0		0		11	(0.11)
Between 2000-2001 and 2009								
			Pulsotype					
			Senft1		BM3			
121	Spring '01	132	1	(0.01)				
121	Summer '09	50	3	(0.06)				
125	Fall '00	50			1	(0.02)		
125	Summer '09	46			9	(0.20)		

Table 21 – Prevalence of *Salmonella* on 18 Michigan dairy farms in 2000-2001 and 2009. AMR and diversity of *Salmonella* from six farms positive for *Salmonella* in both time frames

		Eighteen farms sampled		Six Farms positive in both 2000-2001 and 2009			
Season[1]	Year	No. Farms	Prevalence[2] (No. samples)	No. farms	No. pulsotypes	% resistant	Simpson's Diversity[4]
Summer	2000	2	0.03 (17/582)	1	19	0.84 (16/19)	0.80
Fall	2000	13	0.11 (86/818)	4	90	0 (0/90)	0.60
Winter	2001	17	0.03 (26/973)	2	23	0 (0/23)	0.58
Spring	2001	18	0.03 (47/1554)	3	51	0.08 (4/51)	0.73
Summer	2001	15	0.07 (86/1208)	4	86	0.27 (23/86)	0.80
Summer	2009	18	0.12 (97/830)	6	77	0.01 (1/77)	0.93

[1] Summer (July, August, and September), Fall (Oct, Nov, Dec), Winter (Jan, Feb, Mar), Spring (April, May, June)

[2] Proportion of samples where at least one *Salmonella* isolate was recovered

[3] Proportion of *Salmonella* isolates that were resistant to at least one tested antimicrobial

[4] Simpson's index of diversity calculated based on the number of distinguishable strains using the serotype and PFGE data

Table 22- Differences in the distribution of *Salmonella* serotypes within farms between 2000-2001 and 2009. Values represent the proportion (positive/total) of samples positive for *Salmonella* in cow and calf areas for each serotype

	2000-2001					2009				
Serotype	Farm ID	Calf Area		Cow Area		Farm ID	Calf Area		Cow Area	
Senftenberg	121	0	(0/64)	0.01	(1/203)	121	0.13	(2/16)	0.03	(1/34)
MDR Senftenberg	129*	0.23	(18/78)	0.01	(3/241)	Not recovered				
Typhimurium	Not recovered					114	0.2	(2/10)	0.17	(6/35)
MDR Typhimurium	101*	0.15	(9/62)	0.01	(3/466)	Not recovered				

*Significantly different proportions ($p < 0.05$) of positive samples between cows and calves.

Table 23 – Maximum between-farm similarity of PFGE banding patterns for serotypes isolated from multiple farms within the same year

Serotype	Year	Farm	Frequency	Maximum banding pattern similarity[1]
Bovis Morbificans	2000	111	122	1.0
		114	2	
		125	1	
Muenster[2]	2000	114	73	0.81
		125	14	
		111	1	
Senftenberg	2000	121	1	0.89
		129	22	
Mbandaka[2]	2009	121	5	0.9
		125	3	

[1]Between group similarity calculated using UPGMA clustering method and the dice coefficient of similarity

[2]Between group similarity significantly less than within group similarity ($p<0.05$), based on permuation testing

Table 24- Maximum similarity of PFGE banding patterns for serotypes recovered from the same farms in 2000-2001 and 2009

Serotype	Farm	Year	Frequency	Maximum banding pattern similarity[1]
Montevideo	101	2000	1	0.84
		2009	29	
Bovis-Morbificans	125	2000	1	100
		2009	10	
Senftenberg	121	2001	1	100
		2009	3	

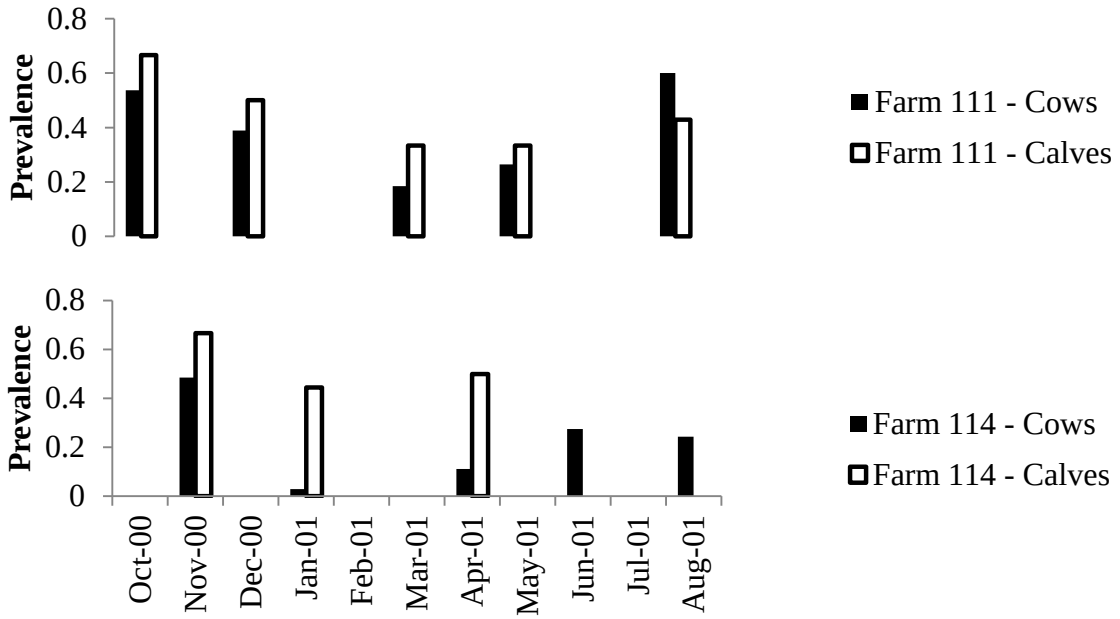
[1]Between group similarity calculated using UPGMA clustering method and the dice coefficient of similarity

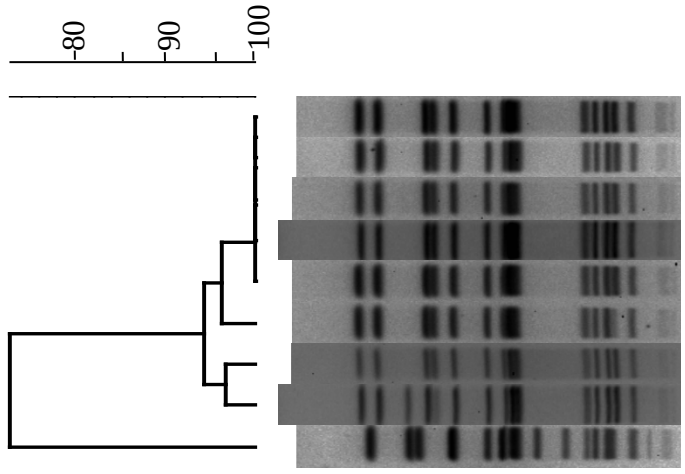
Table 25- Maximum similarity of PFGE banding patterns of serotypes recovered from different farms in 2000 and 2009

Serotype	Farm	Year	Frequency	Maximum banding pattern similarity[1]
Hartford	101	2000	1	
	111	2009	1	0.90
Mbandaka	129	2000	1	
	121	2009	5	0.90
	125	2009	3	0.96
Typhimurium[2]	101	2001	17	
	114	2009	8	0.56

[1]Between group similarity calculated using UPGMA clustering method and the dice coefficient of similarity
[2]Significantly distinct groups, based on permuation testing of the ratio of between group similarity/within group similarity ($p<0.05$)

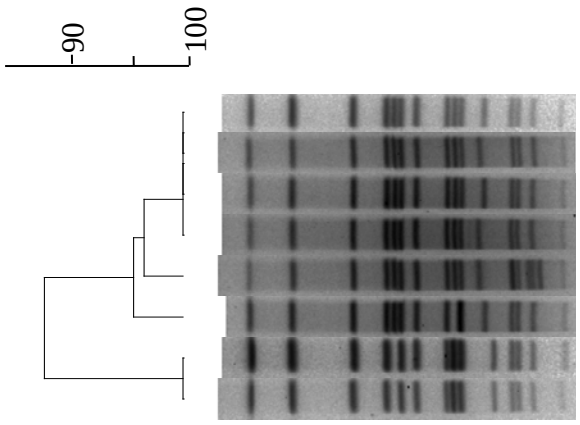
Figure 4. Prevalence over five bimonthly visits in cow and calf samples of the dominant pulsotypes on the herds with the highes prevalence. Top: Farm 111, pulsotype ‘BM3’; Bottom: Farm 114, pulsotype ‘Muens1’. Closed bars, adult cows; open bars, calves.





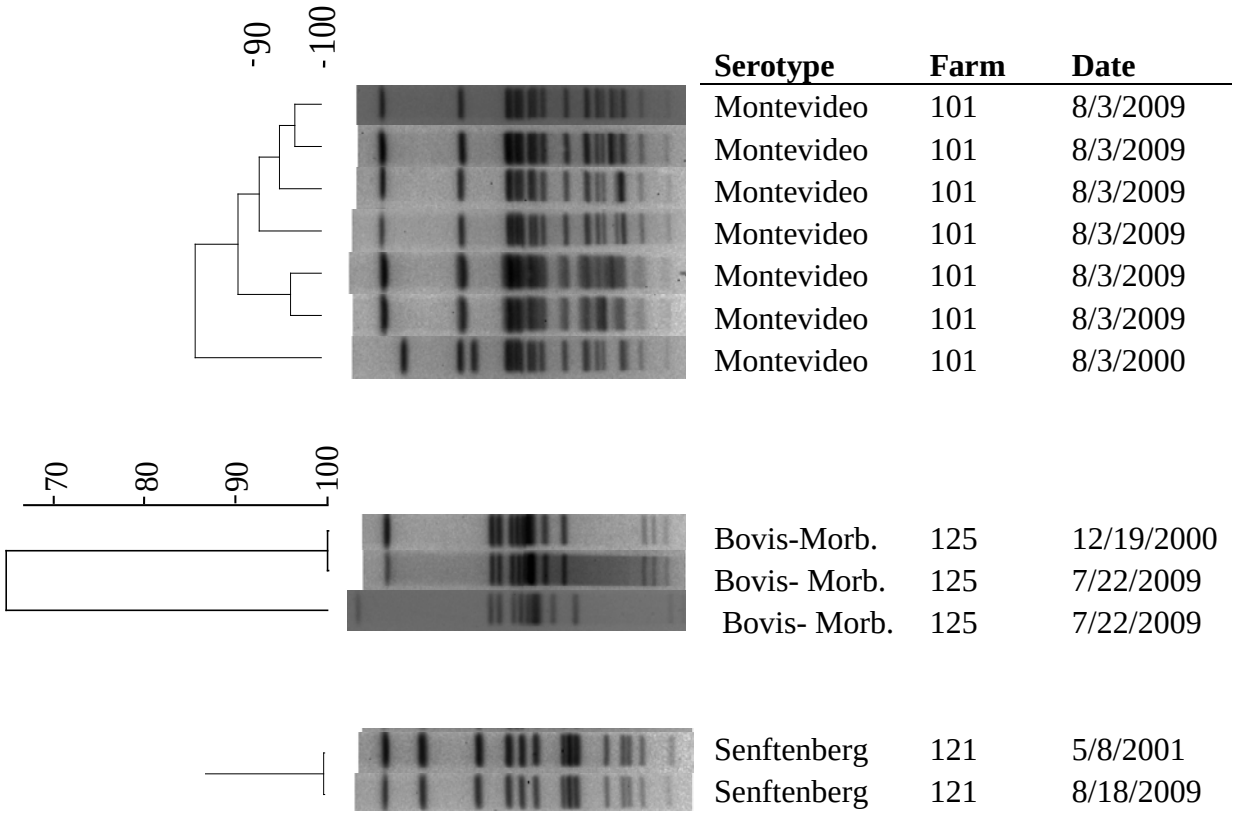
Farm	Date	AMR Profile
101	6/29/2000	ACSSuT
101	7/6/2000	ACSSuT
101	7/13/2000	ACSSuT
101	7/20/2000	ACSSuT
101	7/13/2000	ACSSuT
101	7/3/2000	ACSSuT
101	7/20/2000	ACSuT
101	7/6/2000	ACSSuT
114	7/22/2009	Pan-susceptible

Figure 5 - Unique pulsotypes at each sampling visit of *Salmonella* Typhimurium recovered from Michigan Dairy Farms in 2000 and 2009. The MDR Typhimurium isolates recovered in 2009 were highly distinct from the pansusceptible isolates recovered in 2000.



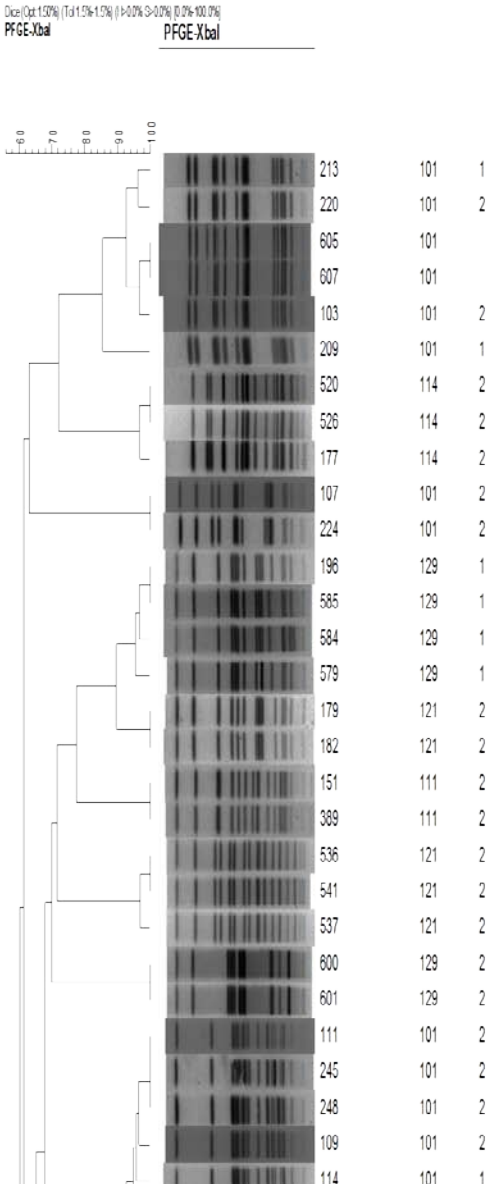
Farm	Date	AMR Profile
129	7/31/2001	GSSuT
129	7/31/2001	GSSuT
129	9/13/2001	GKSSuT
129	9/13/2001	GKSSuT
129	7/31/2001	GKSSuT
129	7/31/2001	GKSSuT
121	5/8/2001	Pan-susceptible
121	8/18/2009	Pan-susceptible

Figure 6 – Unique pulsotypes at each sampling visit of *Salmonella* Senftenberg recovered from Michigan dairy farms in 2001 and 2009. MDR isolates of *Salmonella* Senftenberg were recovered in 2001 but not 2009. Pansusceptible isolates of *Salmonella* Senftenberg from 2001 were indistinguishable from a *Salmonella* Senftenberg isolate recovered from the same farm in 2009



Serotype	Farm	Date
Montevideo	101	8/3/2009
Montevideo	101	8/3/2009
Montevideo	101	8/3/2009
Montevideo	101	8/3/2009
Montevideo	101	8/3/2009
Montevideo	101	8/3/2009
Montevideo	101	8/3/2000
Bovis-Morb.	125	12/19/2000
Bovis- Morb.	125	7/22/2009
Bovis- Morb.	125	7/22/2009
Senftenberg	121	5/8/2001
Senftenberg	121	8/18/2009

Figure 7 - Unique pulsotypes recovered in each sampling year for serotypes that were recovered from the same farms in 2000-2001 and 2009. Indistinguishable strains were recovered from the same farm for serotypes Bovis-Morbificans and Senftenberg.



Farm	Year	Serotype	ST	AMR
101	2000	Typhimurium	19	ACSSu
101	2000	Typhimurium	19	ACSSuT
101	2000	Typhimurium	19	ACSSuT
101	2000	Typhimurium	19	ACSSuT
101	2000	Typhimurium	19	ACSSuT
101	2000	Typhimurium	19	ACSSuT
114	2009	4,5,12:I:-	19	--
114	2009	4,5,12:I:-	19	--
114	2009	Typhimurium	19	--
101	2000	Oranienburg	23	--
101	2000	Oranienburg	23	--
129	2001	Senftenberg	14	GKSSuT
129	2001	Senftenberg	14	GKSSuT
129	2001	Senftenberg	14	GKSSuT
129	2001	Senftenberg	14	GKSSuT
121	2001	Senftenberg	14	--
121	2009	Senftenberg	14	--
111	2001	Anatum	64	--
111	2001	Anatum	64	--
121	2009	Braenderup	22	--
121	2009	Braenderup	22	--
121	2009	Braenderup	22	--
129	2009	Cerro	367	--
129	2009	Cerro	367	--
101	2009	Montevideo	138	--
101	2009	Montevideo	138	--
101	2009	Montevideo	138	--
101	2009	Montevideo	138	--
101	2009	Montevideo	138	--

Figure 8: PFGE patterns and sequence types for *Salmonella* serotypes recovered from Michigan dairy farms in 2000-2001 and 2009. Dendrogram was produced using the dice cofficient of similaritiy and UPGMA clustering algorithms in Bionumerics with a 1.5% optimization. Distinguishable PFGE patterns from each farm in each time point are presented.

Figure 8 (Continued)

Farm	Year	Serotype	ST	AMR
101	2009	Montevideo	138	--
101	2009	Montevideo	138	--
101	2009	Montevideo	138	--
101	2009	Montevideo	138	--
101	2000	Oranienburg	23	--
101	2000	Newport	350	--
101	2000	Hartford	999	--
111	2009	Hartford	405	--
114	2001	Brandenburg	65	--
114	2001	Brandenburg	65	--
114	2001	Give	654	--
114	2001	Give	654	--
111	2009	Thompson	26	--
121	2009	Mbandaka	413	--
121	2009	Mbandaka	413	--
121	2009	Mbandaka	413	--
125	2009	Mbandaka	413	--
129	2001	Mbandaka	413	--
111	2001	Bovis-Morb.	150	--
111	2001	Bovis-Morb.	150	--
125	2000	Bovis-Morb.	150	--
111	2001	Bovis-Morb.	150	--
114	2000	Bovis-Morb.	150	--
114	2000	Bovis-Morb.	150	--
125	2009	Bovis-Morb.	150	--
111	2001	Bovis-Morb.	150	--
111	2000	Bovis-Morb.	150	--
111	2001	Bovis-Morb.	150	--
111	2000	Bovis-Morb.	150	--
114	2001	Meleagridis		--
129	2001	Meleagridis		--
114	2001	Muenster	321	--
125	2001	Muenster	321	--
114	2001	Muenster	321	--
111	2000	Muenster	321	--
114	2001	Muenster	321	T
114	2000	Muenster	321	--
114	2000	Muenster	321	--
125	2009	Bovis-Morb.	150	CxT

CHAPTER FIVE

The Association between Changes in the Population of *Salmonella* and Changes in Antimicrobial Susceptibility

Structured Abstract

Objective: 1) Use principle components analysis to determine differences in susceptibility profiles between serogroups, serotypes and pulsotypes of *Salmonella* recovered from Michigan dairy farms in 2000-2001 and 2009. Determine if observed changes in the antimicrobial susceptibility are associated with changes in the distribution of *Salmonella* serogroups.

Design: Retro-prospective

Sample Population: Eighteen Michigan dairy farms in 2000-2001 and 2009

Procedure: Stored *Salmonella* isolates recovered during a 2000-2001 longitudinal study on Michigan dairy farms were retrieved. Fecal and environmental samples were prospectively collected from the same 18 Michigan dairy farms in 2009 using comparable sample collection techniques. Serogroups, serotypes, and PFGE banding patterns were identified for isolates from 6 *Salmonella*-positive farms in both 2000-2001 and 2009. The MICs for 15 different antimicrobials were determined for all *Salmonella* isolates in each time point. Principle components analysis was used to reduce the dimensions of the data and investigate differences in resistance phenotypes and MIC profiles for serotypes and pulsotypes within and between time frames.

Results: Six farms were positive for *Salmonella* in 2000 and 2009. In total, 271 and 77 isolates were from each year, respectively, were included in the analysis. Principle components plots were useful to show distinct clusters of isolates based on the MIC values to 15 antimicrobials, and showed three distinct clusters representing MDR *Salmonella* Typhimurium with the ACSSuT

resistance phenotype, MDR *Salmonella* Senftenberg with the GKSSuT resistance phenotype, and isolates susceptible to all antimicrobials. Within the cluster of pansusceptible *Salmonella*, however, 2009 isolates tended to cluster separately from susceptible isolates collected in 2000-2001. A second PCA using only non-MDR *Salmonella* showed that the MIC profiles of serogroups E1 and E4 had differing MIC profiles compared to serogroups C1 and C2. Serogroups E1 and E4 were the predominant serogroups in 2000-2001, and serogroups C1 and C2 were the predominant serogroups in 2009.

Conclusions: Results suggest that changes in the prevalence AMR were the result of displacement of MDR strains of Senftenberg and Typhimurium by susceptible strains of the same serotype. Similar within-farm decreases in the MIC_{50} of naladixic acid, chloramphenicol and increase in the MIC_{50} of gentamicin were primarily associated with the disappearance of E1 and E4 serogroups and higher recovery of C1 and C2 serogroups in 2009.

Introduction

Antimicrobial resistance (AMR) in *Salmonella* reduces treatment options for clinicians and veterinarians, and increases the morbidity and mortality of salmonellosis in humans (Maragakis et al. 2008; Varma et al. 2005a). Patients infected with resistant strains were more likely to have septicemia (Varma et al. 2005), and had significantly higher hospital costs relative to patients infected with susceptible strains (Maragakis et al. 2008). In human outbreaks of salmonellosis, 22% of patients infected with AMR strains were hospitalized, while only 8% of patients infected with susceptible strains were hospitalized (Varma et al. 2005).

Antimicrobial use on dairy farms has been postulated to increase resistance in zoonotic pathogens and lead to the emergence of novel strains. Specifically, *Salmonella* Typhimurium DT104 from dairy farms has caused outbreaks and illnesses in people (Glynn et al. 1998), and the emergence of ceftriaxone-resistant *Salmonella* Newport has been attributed to the use of ceftiofur in dairy animals (Zhao et al. 2003).

The prevalence of AMR in the population of *Salmonella* on dairy farms has typically been low. Ray et al., 2007 demonstrated that the majority of isolates (1223/1506) from Midwestern and Northeastern dairy farms were susceptible to all tested antimicrobials, but 24% herds harbored at least one isolate resistant to any antimicrobial. By contrast, studies utilizing clinical isolates have shown higher frequencies of AMR. Indeed, the frequency of AMR in bovine isolates was higher than comparable *Salmonella* serotypes recovered from clinically ill humans (Hoelzer et al. 2010).

National-level estimates of the prevalence of AMR in *Salmonella* from adult dairy cattle have been provided by the NAHMS. In studies done in 1996, 2002, and 2007, 92.3%, 82,3%,

and 96.6% of isolates were susceptible to all of the tested antimicrobials, respectively (USDA 2011b). These studies include only adult cattle, and may therefore underestimate the prevalence of resistance (A. C. B. Berge et al. 2006). Nonetheless, they have shown an important decrease in the prevalence of AMR in *Salmonella* on dairy farms between 2002 and 2007(USDA 2011b).

Temporal changes in the prevalence of AMR in *Salmonella* are most frequently a result of the dissemination of resistant clones or changes in the relative prevalence of specific subtypes (M Davis et al. 2002; Butaye et al. 2006). For instance, a decline in the AMR of *Salmonella* Typhimurium recovered from clinically ill patients in the UK was caused by a concurrent decline in the proportion of *Salmonella* Typhimurium infections that were DT104. This research group has previously described within-farm decreases in the AMR of *Salmonella* recovered from the same Michigan farms in 2000-2001 and 2009, as well as differences in the distribution of serogroups between time frames. The objective of this study was to determine the association between observed susceptibility changes and changes in the population of *Salmonella* on Michigan dairy farms between 2000-2001 and 2009.

Materials and Methods

Salmonella Isolation

Isolation of *Salmonella* was performed in the same laboratory with highly similar protocols in 2000 and 2009 (Fossler et al. 2004). With the exception of a confirmatory step (urea agar used in 2009), and the number of colonies chosen for confirmatory steps (up to five in 2009, and up to two in 2000), the protocols for the isolation and confirmation of *Salmonella* from fecal and environmental samples were identical. Samples were enriched by adding tetrathionate broth as to achieve a 1:10 dilution and incubating for 48 h at 37 °C. The enriched sample was streaked

onto XLT4 agar and incubated for 24 h at 37 °C. In 2009, up to five suspect colonies from XLT4 agar, (red or yellow with black centers) were inoculated onto TSI and urea agar slants, and incubated for 24 h at 37 °C. In 2000, up to two suspect colonies from XLT4 agar, (red or yellow with black centers) were inoculated onto TSI only, and incubated for 24 h at 37 °C. Colonies with test results typical for *Salmonella* (alkaline/acid/H2S positive and urease negative) were then inoculated onto lysine-iron agar and Simmons citrate agar slants. Colonies that were lysine decarboxylase and hydrogen sulfide positive in lysine-iron agar (purple slant and purple-black butt) as well as positive in Simmons citrate (blue) were considered positive for *Salmonella*. *Salmonella* isolates harvested in 2000 were frozen in tryptic soy broth/glycerol solution at −80 °C and stored in cryovials. In 2009, these were retrieved, and underwent further biochemical confirmation before antimicrobial susceptibility testing. Isolates were stabbed onto a TSA slant, and stored at room temperature prior to antimicrobial susceptibility testing.

Antimicrobial Resistance Testing

Salmonella isolates collected in the 2000-2001 study were tested concurrently with the 2009 isolates using the same commercially prepared microbroth dilution antimicrobial panels (CMV7CNCD). This panel contained a prepared range of concentrations for the following 15 antimicrobials: amikacin, amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, naladixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. Isolates frozen in tryptic soy broth in 2001 were regrown by inoculating the isolates onto tryptic soy agar. *Salmonella* recovered from 2009 samples were inoculated onto tryptic soy agar following initial identification. All *Salmonella* from 2000-2001 and 2009 were subsequently streaked to Mueller Hinton agar and incubated for 18–24 h at 37 °C. Testing was performed according to the

instructions from the manufacturer of the automated microbroth dilution system (Trek Diagnostic Systems, Inc.), and panels were read with an autoreader. Quality control tests were performed using E. coli ATCC 25922 for all panels, and were all within acceptable limits. Breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) were used to classify isolates as susceptible, intermediate, or resistant (CLSI, 2010). No CLSI interpretive criteria were available for ceftiofur or streptomycin, so breakpoints presented in the NARMS 2007 Annual Report were used (FDA, 2010).

Serotype Identification

Serotype identification was performed at the DCPAH at MSU using the Kauffman-White scheme. The LPS-associated antigen (O-antigen) and the flagellar associated antigens (H-antigens) were identified using slide and tube agglutination techniques, respectively (23).Where a group of isolates collected from the same farm on the same day had indistinguishable PFGE banding patterns, only one isolate was serotyped, and the remaining isolates are reported as the same serotype.

Pulsed-Field Gel Electrophoresis

Up to two isolates from each fecal or environmental sample underwent PFGE. If more than two isolates were recovered from a single sample, then two isolates were randomly chosen to undergo PFGE. PFGE was conducted at the DCPAH using a CDC standardized protocol (31). Briefly, DNA was prepared and incubated with the XbaI rare-cutting restriction enzyme. The resultant macrorestriction fragments were prepared within 1% agarose gel plugs and separated using pulsed field gel electrophoresis with an initial switch time of 2.2s, final switch time of 63.8s, and a total run length of 16.2 hrs. Gels were stained with 400 mL ethidium bromide

solution and photographed with GelDoc Imager (BioRad). Band patterns were imported into the Bionumerics (v. 4.2 Applied Maths, Kortrijk, Belgium) and standardized against *Salmonella* Braenderup H9812. If two isolates from the same sample had indistinguishable PFGE patterns, then one isolate was included in the statistical analysis and summary statistics.

Statistical Analysis

Co-resistance (the coexistence of genes or mutations conferring resistance to multiple antimicrobials) and cross-resistance (where resistance to one drug also results in resistance to another drug) result in a high degree of correlation between the MICs antimicrobials (1). Given the expected high degree of correlation between MIC values measured on the same isolate, principle components analysis (PCA) was used to reduce the dimensions of the data, and facilitate representations of differences in MIC profiles between years for serotypes and pulsotypes. MIC values for 15 antimicrobials were $\log_2$ transformed, resulting in equivalent distances between sequential 2-fold dilutions of the antimicrobials. An eigenanalysis was performed on the covariance matrix of the $\log_2$ transformed values, resulting in 15 continuous and uncorrelated variables (principle components). The proportion of the total variance of the MIC values for all 15 antimicrobials described by each principle component was calculated. Loadings were calculated to describe the correlation of each principle component with the MIC values of each antimicrobial.

Results

In total, 271 and 77 *Salmonella* isolates were recovered from six farms in 2000-2001 and 2009, respectively. The distribution of serotypes and pulsotypes of *Salmonella* recovered from these farms has previously been described (Chapter 4). Briefly, 11 and 10 different serotypes

were recovered in 2000-2001 and 2009, respectively. Using PFGE, 26 and 38 distinct pulsotypes were recovered in each time frame, respectively. The distribution of serogroups of *Salmonella* from the same Michigan dairy farms was different between years. Serogroup E1 and E4 were significantly more common in 2000-2001 than 2009. Either E1 or E4 was the predominant serogroup on four of the six farms in 2000-2001. In 2009, serogroup E1 was not recovered, and only 3 isolates of E4 were recovered from a single farm in 2009. Serogroup C1 increased in prevalence between 2000-2001 and 2009, and was the predominant serogroup on 3 of the 6 farms in 2009.

Within-farm changes in the susceptibility of the *Salmonella* isolates have also been previously described . The resistance phenotypes of serotypes recovered in 2000-2001 and 2009 were different across years. MDR strains of *S.* Typhimurium and *S.* Senftenberg were recovered from farms 101 and 129 in 2000 and 2001, respectively. In 2009, pansusceptible strains of Typhimurium and Senftenberg were recovered from different farms (**Table 18**). One isolate each of Bovis-morbificans were resistant to streptomycin or sulfisoxazole in 2001, and ceftriaxone and tetracycline in 2009.

There were also changes in the MIC_{50} that were not explained by the presence or absence of MDR strains. The 2009 MIC_{50} was lower relative to 2000 or 2001 for nalidixic acid, chloramphenicol, and sulfisoxazole, and higher for gentamicin. Similar changes in MIC's were distributed across a majority of farm. Four of six farms each had similar decreases in the MIC_{50} for nalidixic acid and chloramphenicol, and 3 of 6 farms had an increase in the MIC_{50} for

gentamicin. Although the change wasn't reflected by changes in the MIC_{50}, 5 of 6 farms had a decrease in the MIC_{50} for sulfisoxazole.

Principle Components Analysis

There was a large degree of correlation between the MICs values of different antimicrobials. The proportion of the variance represented by the first five principle components (PCs) was 0.46, 0.28, 0.08, 0.06, and 0.04. Loading values for the first PC showed larger absolute values for antimicrobials that are components of the ACSSuT and GKSSuT phenotypes that were found in strains of Typhimurium and Senftenberg (**Table 27**). Decreasing values on the X axis of the plot of PCs 1&2 (**Figure 9**) correspond with increasing levels of AMR. MDR strains of Typhimurium (**Figure 9**, top left) and Senftenberg (**Figure 9**, bottom left) clustered separately from the remaining pansusceptible *Salmonella* isolates recovered in both 2000-2001 and 2009. Changes in the AMR phenotypes based on resistance categorizations between years were a result of changes in pulsotypes within serotypes. However, as previously noted, there were differences in the MIC_{50} between years that were not explained by the presence or absence of MDR strains. Additionally, non-MDR isolates from 2009, tended to cluster separately from non-MDR isolates from 2000 or 2001 (**Figure 9**).

Therefore, a second PCA was conducted to explain differences in MICs between years that weren't explained by the presence or absence of strains categorized as resistant. For the second PCA, the proportion of the variance explained by the first five PCs was 0.31, 0.24, 0.15, 0.09, and 0.06. Eighty percent of the total variance was explained the first four principle components. Loadings for the first PC had the largest negative values for chloramphenicol,

cefoxitin, sulfisoxazole, ceftiofur, and nalidixic acid, and the highest positive values for gentamicin (**Table 28**). Loadings for PC2 had the largest negative values for gentamicin and cefoxitin and the largest positive values for sulfisoxazole and trimethoprim-sulfa. Decreasing (more negative) values for the X and Y axis of the plots of the first and second PC are therefore useful to depict between-year changes in the MIC_{50} that were previously described. Subsequent tables (**Table 29**, **Table 30**) present those antimicrobials with differences in the MIC_{50} between years, or have the highest loading for PC1. Points in the lower left of the plot reflect increasing susceptibility to chloramphenicol, cefoxitin, sulfisoxazole and ceftiofur, and decreasing resistance to gentamicin. The MIC profiles of isolates from 2009 generally had distinct MIC profiles relative to isolates from 2000 or 2001; however, there was significant amount of overlap (**Figure 10**). The results presented below explore whether the between-year differences in susceptibility were a consequence of changes in the population of serogroups, serotypes, or pulsotypes.

Serogroups

The PC plots of the 2nd PCA demonstrated that serogroups E1 and E4 clustered separately from other pansusceptible serogroups within both time frames (**Figure 11**). Within the 2000-2001 time frame, serogroup C2 *Salmonella* had a lower MIC_{50} for chloramphenicol and cefoxitin than serogroup E1 and/or E4 strains (**Table 29**). Within the 2009 time frame, serogroup C2 *Salmonella* had a lower MIC_{50} for chloramphenicol, cefoxitin, and naladixic acid (**Table 29**).

Excluding MDR strains, there were decreases in the MIC_{50} within serogroups C1, C2, and B between years (**Table 29**). The MIC_{50} of chloramphenicol and cefoxitin for serogroup C1 strains was lower in 2009 than 2000-2001. This difference was associated with differences in MICs between serotypes that were recovered in either time frame. The most common serogroup C1 serotype in 2000-2001 (Oranienburg) clustered separately from the most common serogroup C1 serotype (Montevideo) recovered in 2009 (**Figure 12**). *S.* Oranienburg had a lower MIC_{50} for chloramphenicol, cefoxitin, and naladixic acid relative to *S.* Montevideo. Likewise, plots of PC1&2 showed that serotype Brandenburg (serogroup B, recovered in 2000) clustered separately from serotypes Typhimurium and 4,5,12:i- (serogroup B, recovered in 2009) (**Figure 12**). *S.* Brandenburg had a higher a MIC_{50} for chloramphenicol, cefoxitin, sulfisoxazole, ceftiofur, and naladixic acid relative to serogroup B serotypes (Typhimurium and 4,5,12:i:-) recovered in 2009.

Serotypes

Six serotypes were recovered in both years (Table 1). Greater than one isolate was recovered for three of the serotypes, including Senftenberg, Typhimurium, and Bovis-morbificans. Differences in the MIC_{50} between years for Senftenberg and Typhimurium were primarily associated with the presence of MDR resistance in 2000 or 2001. However, there were smaller decreases in the MIC_{50} within serotype Bovis-morbificans (serogroup C2) for sulfisoxazole and nalidixic acid (**Table 30**).

Pulsotypes

Considering all serotypes, a total of 64 distinguishable pulsotypes were identified in either 2000-2001 or 2009. The most common pulsotypes recovered were pulsotypes of Bovis-morbificans and Muenster recovered in 2000-2001. Within time frames, the MIC profiles of isolates with indistinguishable PFGE banding patterns did not appear to cluster (**Figure 13**). Two pulsotypes were recovered in both years; indistinguishable strains of Bovis-morbificans and Senftenberg were recovered in both 2001 and 2009. Only a single isolate of Senftenberg was recovered within farm 121 in 2001. However, 116 and 9 isolates of a pulsotypes of Bovis-morbificans were recovered in 2001 and 2009, respectively. Between years, the MIC_{50} for the same pulsotypes was lower for sulfisoxazole and naladixic acid, and higher for gentamicin.

Discussion

This study utilized a retro-prospective study design to assess changes in the AMR profiles and shifts in the distribution of MICs for *Salmonella* recovered in 2000-2001 and 2009. The results demonstrate changes in the resistance phenotypes of Typhimurium and Senftenberg, as well as differences in the MIC_{50} between years that were mostly attributable to changes in the distribution serogroups and serotypes between years. Changes in the prevalence of AMR documented by this study are consistent with results from consecutive national-level cross-sectional studies by NAHMS. The proportion of isolates that were resistant to two or more antimicrobials was 5% and 2.7% in 2007 and 2002, respectively (USDA 2011b).

Limitations of this study include the small number of farms that were positive for *Salmonella* at both time points. Larger studies will be required to extrapolate these results to a broader population of dairy farms or to investigate management practice associations with

changes in AMR. Additionally, differences in the laboratory handling of isolates from 2000-2001 and 2009 may account for some differences in MIC profiles between years. Retrospective isolates had been frozen for approximately 10 year prior to susceptibility testing. Isolates from 2009 were tested immediately after identification and prior to freezing. Freezing had been reported to increase the susceptibility of *Campylobacter* isolates (Humphrey & Cruickshank 1985). However, the freeze/regrowth stress applied to the 2000-2001 *Salmonella* isolates and not the 2009 isolates would have been expected to have the opposite effects to those demonstrated in this study. Furthermore, similar differences in MIC profiles between serogroups were seen within each year, where *Salmonella* were subjected to identical laboratory procedures. Nonetheless, the effects of differences in laboratory techniques cannot completely be ruled out.

Principle components analysis was useful in this study to reduce the dimensions of the data and demonstrate differences in the MIC profiles between *Salmonella* types. Multivariate statistical techniques have been applied in previous studies of AMR. Principle components and discriminant function analysis were used to demonstrate large differences in the susceptibility profiles of environmental isolates from different species of livestock (Kaneene et al. 2007). Additionally, Berge et al., 2003 used disk diffusion data to identify clusters of AMR in E. coli recovered from calves. Multivariate analysis has also been applied to the binary resistance categorizations to identify patterns in swine and people living in close proximity (Alali et al. 2008).

Results from this study are consistent with shifts in AMR due to shifts in the population of *Salmonella*. Specifically, displacement of MDR pulsotypes by susceptible pulsotypes was associated with changes in the prevalence of AMR. Between-year changes in the distribution of MICs were associated with changes in the population of serogroups and serotypes. Within

serogroups C1 and B, serotypes recovered only in 2009 had different MIC profiles than serotypes recovered in 2000-2001. Differences in the MIC profiles among susceptible *Salmonella* suggest differences in the intrinsic susceptibility across these serogroups and serotypes.

Only two pulsotypes were recovered in both 2000-2001 and 2009. For the pulsotypes of Senftenberg, only a single isolate was recovered in 2000-2001. Therefore, the change in susceptibility within pulsotypes between years was difficult to assess. Nonetheless, for the pulsotypes of Bovis-morbificans, the MIC_{50} for sulfisoxazole and naladixic acid was lower in 2009, and the MIC_{50} for gentamicin was higher in 2009. These are similar differences in susceptibility compared to between serogroups within each year. The pulsotypes of Bovis-morbificans were recovered from different farms in each time frame. Differences in susceptibility may be attributable to differences in farm management practices or antimicrobial use.

Similar increases and/or decreases in MICs were distributed across a majority of farms. Similar changes in AMR across farms appeared to be a result of similar changes in the population of *Salmonella* between 2000-2001 and 2009, and differences in the intrinsic susceptibility of Salmonella to the tested antimicrobials. Previous research provides several examples where population changes or clonal displacement resulted in changes in prevalence estimates for AMR in *Salmonella*. For instance, the proportion of *Salmonella* resistant to ceftiofur increased relatively rapidly between the years 1999 and 2006 (FDA 2010). These increases were largely the result of the dissemination of clonal MDR *Salmonella* Newport. The emergence of DT104 in the 1990's caused increases in the prevalence estimates of AMR for

certain antimicrobials (Threlfall et al. 2006) and was associated with the displacement of chloramphenicol susceptible *Salmonella* Typhimurium strains from a diagnostic laboratory (Davis et al. 1999). After the frequency of illnesses caused by DT104 peaked in the 2000's, more recent evidence indicates that this strain is becoming less prevalent (40, 41). Decreases in AMR of *Salmonella* in consecutive cross-sectional studies by NAHMS were particularly notable for antimicrobials that are components of the ACSSuT phenotype, which has most frequently been associated with *S.* Typhimurium DT104. The 2007 NAHMS Dairy study was the first study where MDR *Salmonella* Typhimurium was not recovered. These results support previous research showing declines in prevalence of MDR subtypes, including *Salmonella* Typhimurium DT104.

Changes in the characteristics and/or practices on livestock farms may result in changes in the population of *Salmonella*. Continued adoption of new practices or technologies may alter the microbial environment and allow *Salmonella* to proliferate, or provide a selective advantage for certain serogroups of *Salmonella*. Specifically, utilization of antimicrobials in the feed which are dispersed to large numbers of animals on the farm, allow AMR *Salmonella* to disseminate more freely. Notable changes in U.S. dairy farms between 2000 and 2009 included increases in herd sizes, and adoption of newer technologies associated with larger herd sizes, such as manure handling practices and housing. Similar within-farm changes in the population of *Salmonella* on these six farms suggest environmental changes that favor serogroup C1 *Salmonella* over E1 or E4. Nonetheless, the number of farms that were positive for *Salmonella* at both time points did not sufficient sample size to analyze the association of management practice changes and shifts in AMR.

Conclusions

In conclusion, within-farm shifts in the population of *Salmonella* resulted in changes in the prevalence of AMR and shifts in the distribution of MICs. Changes in the prevalence AMR were the result of displacement of MDR strains of Senftenberg and Typhimurium by pansusceptible strains of the same serotype, while shifts in the distribution of MICs among the pansusceptible populations of *Salmonella* were caused primarily by the disappearance of E1 and E4 serogroups, and higher frequency of recovery of serogroup C1. Principle components analysis was useful to depict clear differences serogroups and serotypes of *Salmonella* recovered in 2000-2001 and 2009.

Table 26 - AMR profiles of *Salmonella* serogroups and serotypes recovered in both 2000-2001 and 2009

Serotype	**2000-2001**		**2009**	
	Profile	Frequency	Profile	Frequency
Serogroup B				
Typhimurium	ACSSuT	15	Susceptible	8
	ACSSu	1		
	ACSuT	1		
Serogroup C1				
Hartford	Susceptible	1	Susceptible	1
Montevideo	Susceptible	1	Susceptible	29
Mbandaka	Susceptible	1	Susceptible	8
Serogroup C2				
Bovis-	Susceptible	123	Susceptible	9
	S	1	CxT	1
	Su	1		
Serogroup E4				
Senftenberg	Susceptible	1	Susceptible	3
	GKSSuT	13		
	GSSuT	4		
	GSuT	2		
	SuT	2		
	GKSuT	1		
Total		168		59

A, ampicillin; C, chloramphenicol; Cx, ceftriaxone; G, gentamicin; K, kanamycin; S, streptomycin; Su, sulfisoxazole; T, tetracycline

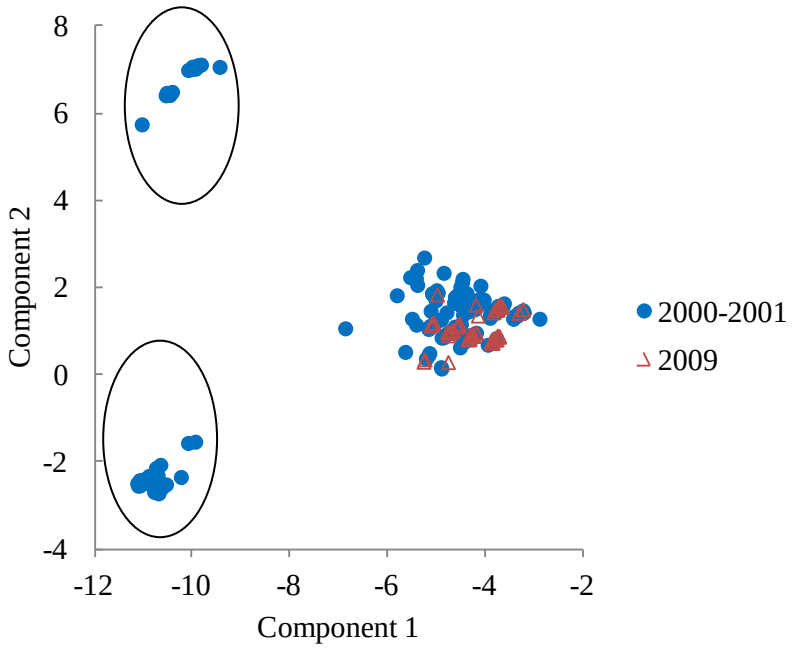


Figure 9 - Principle components 1 and 2 using the MICs for all 15 antimicrobials. Top and bottom circles represent MDR strains of Typhimurium and Senftenberg, respectively, recovered in 2000-2001. The third group represents predominantly pansusceptible isolates, or isolate resistant to only tetracycline (n=1), streptomycin (n=1), or sulfisoxazole (n=1).

Table 27 - Principle component loadings and changes in AMR between 2000-2001 and 2009 for all *Salmonella* isolates. Table is sorted by the loading for the first principle component.

Antimicrobial	**PC1 Loading**	**Number (%) resistant**		**MIC_{50}**	
		2000-2001	2009	2000-	2009
Tetracycline	-0.948	40 (14.9)	1 (1.3)	4	4
Streptomycin	-0.871	33 (12.3)	0 (0)	32	32
Sulfisoxazole	-0.857	39 (14.5)	0 (0)	32	32
Kanamycin	-0.732	14 (5.2)	0 (0)	8	8
Gentamicin	-0.731	20 (7.4)	0 (0)	0.25	0.5
Choramphenicol	-0.600	16 (5.9)	0 (0)	8	4
Amox-Clav	-0.578	0 (0)	0 (0)	1	1
Ampicillin	-0.576	16 (5.9)	0 (0)	1	1
Trimeth-Sulfa	-0.544	0 (0)	0	0.12	0.12
Cefoxitin	-0.227	0 (0)	0 (0)	2	2
Ceftiofur	-0.174	0 (0)	0 (0)	1	1
Amikacin	-0.161	0 (0)	0 (0)	1	1
Naladixic Acid	-0.084	0 (0)	0 (0)	4	2
Ceftriaxone	0.002	0 (0)	1 (1.3)	0.25	0.25
Ciprofloxacin	0.014	0 (0)	0 (0)	0.015	0.015

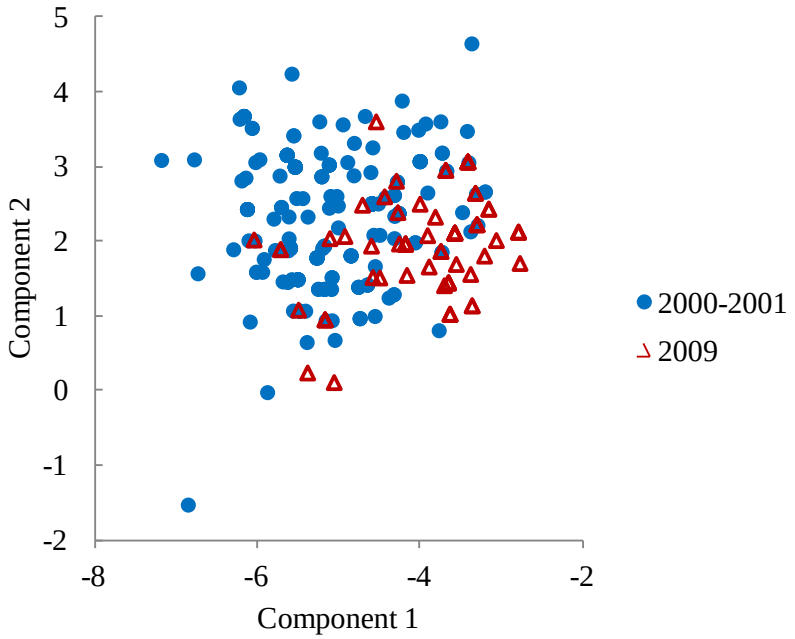


Figure 10 - Principle components 1 and 2 using the MICs for all 15 antimicrobials and only non-MDR *Salmonella*. Closed circles = isolates from 2000-2001. Open triangles = isolates from the same farms in 2009.

Table 28 - Principle component loadings and changes in AMR between 2000-2001 and 2009 for all *Salmonella* isolates. Table is sorted by the loadings for the first principle component.

Antimicrobial	Loadings		MIC_{50}		Frequency of Farms		
	PC1	PC2	2000-2001	2009	Inc	Dec	No Change
Chloramphenic	-0.761	-0.044	8	4	0	**4**	**2**
Cefoxitin	-0.706	-0.598	2	2	0	**6**	**0**
Sulfisoxazole	-0.657	0.568	32	32	0	**4**	**2**
Ceftiofur	-0.576	-0.210	1	1	0	**2**	**4**
Naladixic Acid	-0.561	0.195	4	2	0	**4**	**2**
Trimeth-Sulfa	-0.306	0.430	0.12	0.12	0	0	6
Ampicillin	-0.216	-0.141	1	1	0	0	6
Amox-Clav	-0.186	-0.105	1	1	0	0	6
Ciprofloxacin	-0.096	0.055	0.015	0.01	0	0	6
Tetracycline	-0.055	-0.065	4	4	0	0	6
Streptomycin	-0.006	0.057	32	32	0	0	6
Ceftriaxone	0.022	0.199	0.25	0.25	0	0	6
Amikacin	0.028	-0.574	1	1	1	0	5
Gentamicin	0.153	-0.605	0.25	0.5	4	0	2
Kanamycin	--	--	8	8	0	0	6

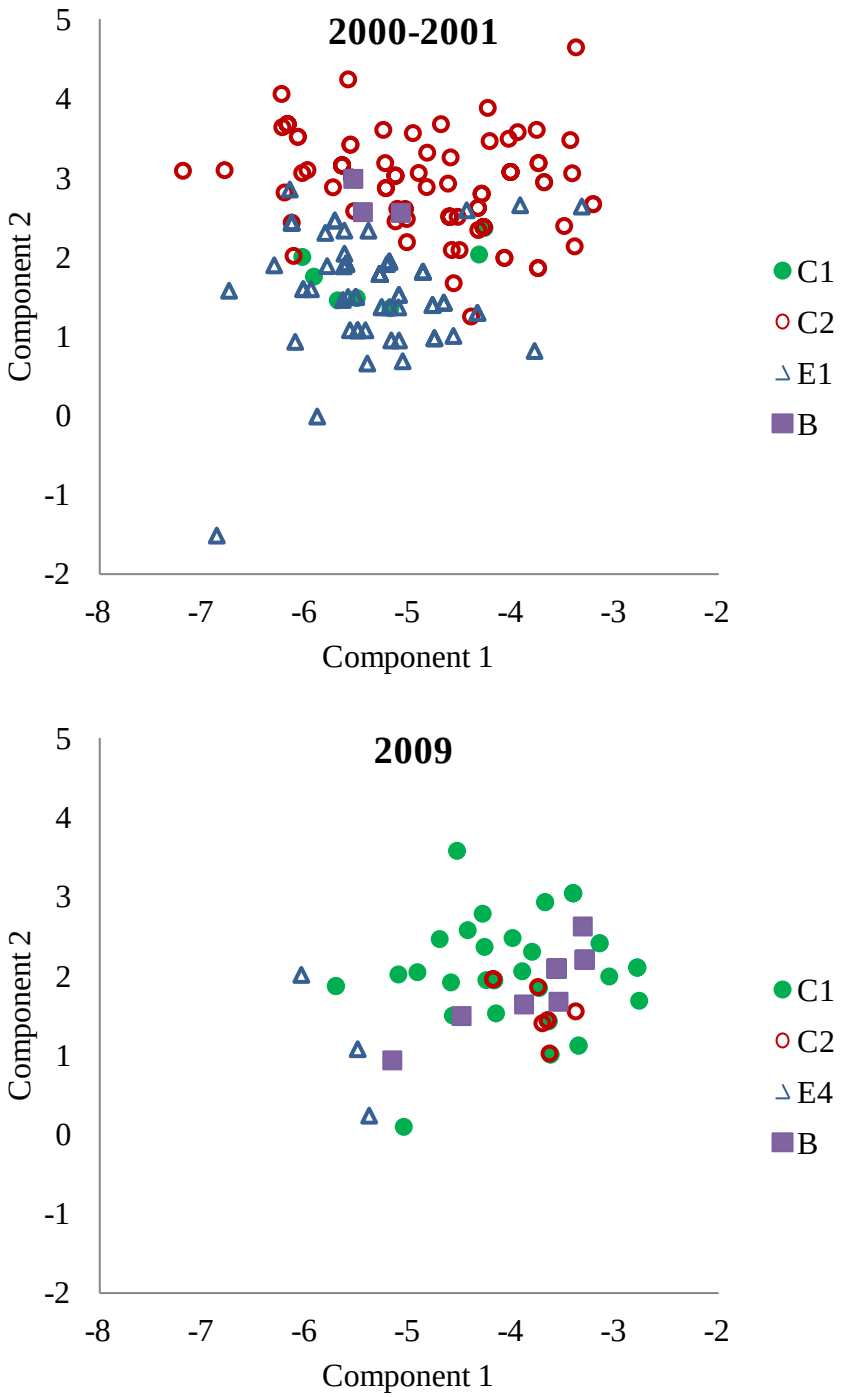


Figure 11 - Principle components 1 and 2 using the $\log_2$ MICs for all 15 antimicrobials and only non-MDR *Salmonella*. Susceptibility varies by serogroup in each time point. Serogroups E1 and E4 are less susceptible than serogroups C1 and C2.

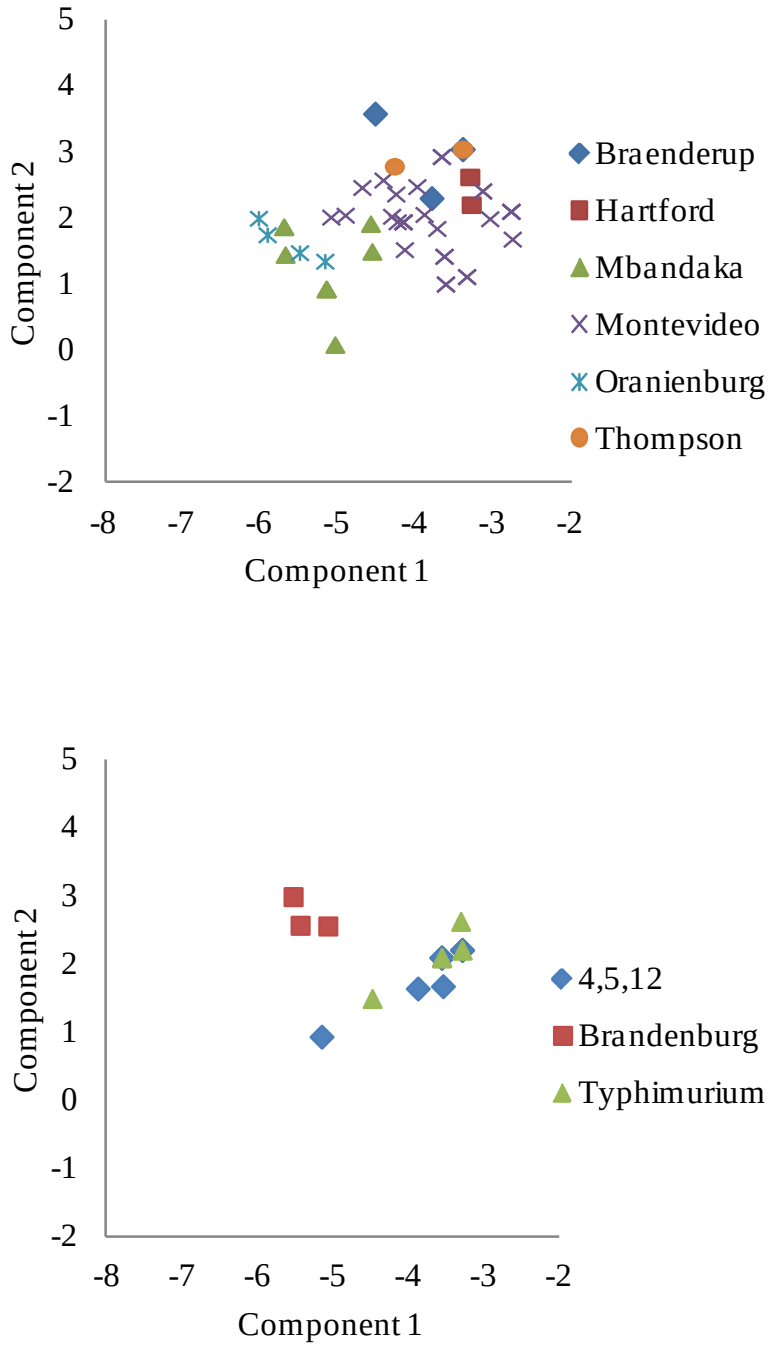


Figure 12 - Principle components 1 and 2 using the $\log_2$ MICs for all 15 antimicrobials and serogroup C1 (top) and serogroup B (bottom). Although there is significant overlap, MIC profiles of the same serotype are generally similar to one another. Serotypes recovered in 2009 are generally more susceptible the serotypes recovered in 2000 or 2001.

Table 29 - MIC_{50} of non-MDR isolates for each serogroup recovered in 2000-2001 or 2009. Antimicrobials presented are those with differences in the overall MIC_{50} between years, and those with the highest loadings from the PCA.

	Number of isolates		**MIC_{50}**											
			Chloramphen		Cefoxitin		Sulfisox		Ceftiofur		Nalidixic Acid		Gentamicin	
	2000-2001	2009	2000-2001	2009	2000-2001	2009	2000-2001	2009	20	2009	2000-2001	2009	2000-2001	2009
B	19	16	8	4	2	1	64	32	1	1	4	2	0.5	0.5
C1	7	46	8	4	4	2	32	32	1	1	2	2	0.5	0.5
C2	125	10	4	4	2	2	64	16	1	1	4	2	0.25	0.5
E1	95	0	8	--	4	--	32	--	1	--	4	--	0.25	--
E4	23	3	8	8	4	4	64	32	1	1	4	4	0.25	0.5
K	0	2	--	4	--	1	--	32	--	0.5	--	2	--	0.63
	269	77												

Table 30 - MIC_{50} for four antimicrobials for *Salmonella* serotypes with greater than one isolate recovered from Michigan dairy farms in 2000-2001 and 2009. The antimicrobials presented are those where the overall MIC_{50} was different between years, or those with the highest loadings for PC1.

	No. of isolates		**Chloramph**		**Cefoxitin**		**Sulfisox**		**Ceftiofur**		**Naladixic**		**Gentamicin**	
Serotype	2000-2001	2009	2000-2001	2009	2000-2001	2009	2000-2001	2009	2000-2001	2009	2000-2001	2009	2000-2001	2009
Bovis-Morbificans	124	10	4	4	2	2	64	16	1	1	4	2	0.25	0.5
Senftenberg	23	3	8	8	4	4	256	32	1	1	4	4	16	0.5
Typhimurium	16	8	32	4	2	1	256	32	1	1	2	2	0.25	0.5

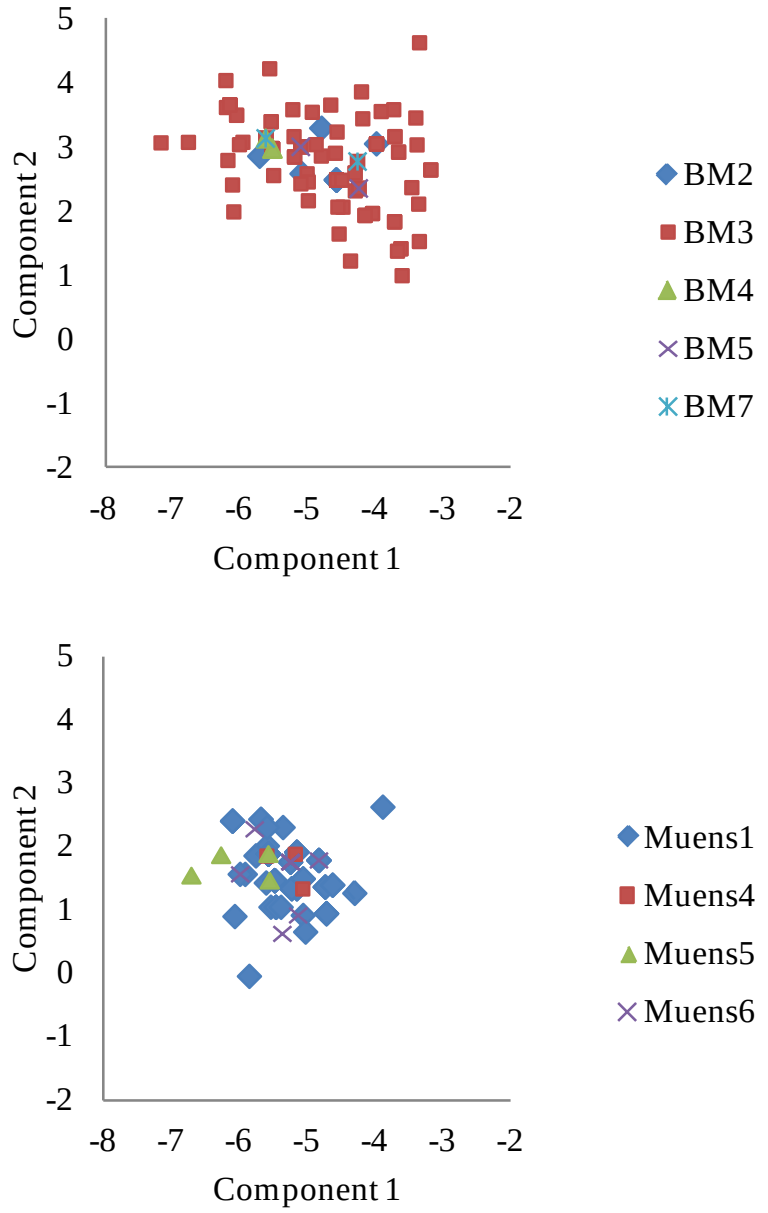


Figure 13 - Principle components 1 and 2 using the $\log_2$ MICs for all 15 antimicrobials and distinguishable pulsotypes of Bovis-Morbificans (top) and Muenster (bottom). Susceptibility is variable within indistinguishable pulsotypes of *Salmonella*, and distinct pulsotypes do not appear to cluster separately.

CHAPTER SIX

Overall Discussion, Conclusions, and Recommendations for Future Research

A fundamental goal in preharvest food safety is to identify mechanisms and processes for population changes in the *Salmonella* on livestock farms that might drive similar changes in humans. Applications of molecular subtyping techniques have improved our knowledge of the diversity and epidemiology of *Salmonella* on dairy farms. Ultimately, these tools may allow more accurate identification of interventions to decrease the prevalence and AMR of *Salmonella* within the United States. Based on the literature review, short term longitudinal studies have shown that the population of *Salmonella* within dairy farms is often quite stable. Data from 2000-2001 study showed important seasonal fluctuations, but the predominant population of *Salmonella* most frequently did not change within farms over a one-year time period. Eleven high-prevalence herds in 2000-2001 were most frequently infected with a single serogroup, and the predominant serogroup did not over the course over a year of sampling (Fossler et al. 2004). Thus, short-term longitudinal studies alone may be insufficient to understand the frequency and drivers of *Salmonella* population changes within farms. A review of the literature did not find instances where long-term within-farm changes in the population and AMR of *Salmonella* have been assessed.

In order to build on previously conducted research, the current study was designed as a retro-prospective study to determine within-farm changes in the prevalence, AMR, and genetic subtypes of *Salmonella* within-farms over a 10-year time frame. We compared the prevalence of *Salmonella* across seasonally matched sampling visits in 2000-2001 and 2009. Overall, the prevalence was higher in 2009 relative to 2000-2001, and there were significant within-farm

increases in prevalence of *Salmonella* in cow or environmental samples for 3 of the 18 farms, and a significant decrease in prevalence of *Salmonella* in cow samples for 1 farm, and a decrease in prevalence of *Salmonella* in calf samples for one farm. Within-farm increases in *Salmonella* prevalence were associated with herd size increases, and tended to be associated with management practice changes. These results are consistent with previously described associations between *Salmonella* prevalence and herd size (Fossler et al. 2004; Blau et al. 2005; Ruzante et al. 2010), and short-term longitudinal studies of *Salmonella* shedding on dairy farms Fossler et al. 2005a). However, the associations with management practices were statistically tenuous, and a greater number of herds is required to have sufficient statistical power to determine the association with management practice changes and changes in *Salmonella* prevalence.

The data on changes in AMR suggest overall decreases in the susceptibility between time frames on the majority of farms. Two different types of changes in the susceptibility of *Salmonella* were identified in this study. First, large decreases in the magnitude of MIC's, were documented only on two farms, and were a result of the recovery of MDR strains in 2000-2001, but not 2009. Second, there were shifts in the distribution of MICs within the susceptible population of *Salmonella* that were distributed across a majority of farms.

Large magnitude differences in the MIC's between years were a result of the recovery of MDR strains in 2000-2001 and not 2009. Rather, pansusceptible strains of the same serotype were recovered. The literature provides examples of clonal displacement within serotypes, where chloramphenicol resistant MDR *Salmonella* Typhimurium displaced chloramphenicol susceptible *Salmonella* Typhimurium (Davis et al. 1999). Alternatively, MDR strains may have lost AMR determinants over the 10 year time frame. Examples of acquisition of phenotypic

resistance within a single PFGE clade have been reported (Davis et al. 2007; Hoelzer et al. 2010). Small differences of PFGE profiles associated with acquisition of phenotypic resistance have also been reported (Soyer et al. 2010). Additional sequencing of the strains may be required to identify the phylogenetic relatedness of MDR and non-MDR strains recovered in this study. However, the large differences in PFGE banding patterns suggest displacement of MDR strains by pansusceptible strains.

Smaller magnitude shifts in the distribution of MICs within the susceptible population were associated with shifts in the population of serogroups. Principle components analysis was useful to identify and illustrate differences in the intrinsic susceptibility patterns of different serogroups and serotypes. The overall distribution of serogroups was significantly different between time frames, reflecting a higher prevalence of serogroup C1, and a lower prevalence of serogroups E1 and E4. Similar shifts towards serogroup C1 were seen on three of the six farms. Prior data on serotype shifts within farms is limited, however the proportion of *Salmonella* recovered in most recent NAHMS surveys reflect similar trends (USDA 2011b). Alternatively, these trends may reflect regional changes in the management practices of dairy farms, rather than national-level changes in the *Salmonella* population. Given the fundamental differences in cell wall structure between serogroups, it is plausible that some practices may provide selective advantages for specific serogroups. The majority of serogroup C1 isolates recovered in 2009 were transiently recovered in 2000-2001, often times from only a single sample. The similarity of PFGE banding patterns for serogroup C1 serotypes was high. Taken together, these results suggest that serogroup C1 serotypes recrudesced after maintaining low-prevalence herd infections for long periods of time. Changes in the microbial environment may have changed to provide a selective advantage to serogroup C1.

The small-magnitude differences in the MIC_{50} between years would not likely impact the outcome of treatment of Salmonellosis. Nonetheless, the differences may represent gradual shifts in the susceptibility of *Salmonella* over time. One-dilution differences in MIC measurements can be caused by variations in laboratory techniques, particularly the total number of bacteria inoculated onto the Sensititre plate. However, only systematic differences between the 2000-2001 and 2009 isolates would explain the statistically significant differences in the distribution of MIC's between time frames. Isolates from either time frame were tested concurrently on the same Sensititre machine, in the same laboratory, and by the same technician. Still, isolates from 2000-2001 were subjected to freezing for 10 years prior to regrowth and susceptibility testing, whereas isolates from 2009 had never experienced freezing. Freeze stress nevertheless would be expected to increase susceptibility (Humphrey & Cruickshank 1985), and these results show that MICs were lower for the 2009 isolates, which never underwent freezing.

Overall, the relatedness of *Salmonella* between-years was high. *Salmonella* serotypes recovered in both time frames had identical gene sequences for seven housekeeping genes. PFGE band patterns of serotypes recovered in both years was high, and in two cases were indistinguishable. The high similarity of gene sequences and PFGE banding patterns suggests long-term persistence and relatively little diversification of this *Salmonella* population over this ten year time frame. The largest difference in the similarity of PFGE banding patterns within serotypes was between MDR and non-MDR strains. Between MDR and non-MDR strains of Typhimurium and Bovis-morbificans, the similarity was less than 60%, whereas between susceptible strains of the same serotype, the similarity of banding patterns was frequently >90%. This is consistent with prior reports, where *Salmonella* Cerro recovered over a 20 year time frame had a single predominant pulsotype (Hoelzer et al. 2011). Chromosomal AMR gene

insertions or acquisition of plasmids carrying AMR genes could cause up to a 3-band difference in the PFGE pattern (Tenover et al. 1995). However, there were more than 3-band differences between MDR and non-MDR strains of the same serotype, suggesting that the MDR strains were not close descendants of the susceptible strains of the same serotype. Otherwise, multiple genetic events occurred concurrently with acquisition of AMR, leading to large differences between PFGE patterns.

Overall Conclusions

This research utilized a unique study design to assess long-term within-farm shifts in the population of *Salmonella* and the association with population changes with changes in AMR. Between time points, within-farm changes resulted in higher prevalence, higher diversity, and lower AMR of *Salmonella* from 2009 relative to 2000-2001. Utilization of serotyping and molecular subtyping techniques showed that pulsotypes of *Salmonella* persisted within farms, and the overall relatedness of *Salmonella* between time periods was high. Differences in the prevalence of MDR strains of *Salmonella* were associated with changes in the population of pulsotypes, and smaller-magnitude increases in susceptibility were associated with shifts in the distribution of serogroups between years. These data significantly add to the knowledge of the epidemiology and ecology of *Salmonella* on dairy farms.

Recommendations for Future Research

The findings of this study open doors to new possibilities in understanding the ecology and epidemiology of *Salmonella*. The design of this study enables a long term assessment of the within-farm, between farm, and between-year changes in genotypes and antimicrobial resistance of *Salmonella*. Based on the results of this study, population displacement may play a significant

role in the observed changes in antimicrobial resistance and prevalence of *Salmonella* in the United States. It is possible that similar mechanisms are responsible for displacement of MDR resistant subtypes on the national-level, and explain decreases in antimicrobial resistance observed in prior NAHMS studies. However, the number of farms that were positive for *Salmonela* in both time frames in this study limits the scope of the inferences. Comparable studies with similar temporal separation and a larger number of herds are necessary to infer mechanisms for population changes at the national-level.

Additionally, a larger number of herds would be useful to assess associations between population changes and changes in farm characteristics and management practices. It is possible, and perhaps likely, that certain management practices selectively favor certain serogroups or sequence types of *Salmonella*. Similar changes in the technology and management practices on U.S. Dairy herds over time may explain the observed population changes in this study; however, the number of herds severely limited to quantitatively assess associations of prevalence and antimicrobial resistance changes with changes in management practices.

Furthermore, a greater number of sampling points within the ten-year time frame would be useful to understand temporal trends in better detail. This study assesses the prevalence, antimicrobial resistance, and the genotypes of *Salmonella* at only two time points.

APPENDICES

APPENDICES

Table 31- Gene sequence of primers used for PCR amplification and sequencing for the seven housekeeping genes used in the MLST protocol

	PCR Amplification	PL[1]	Sequencing
thrA	F 5'-GTCACGGTGATCGATCCGGT-3' R 5'-CACGATATTGATATTAGCCCG-3' R1 5'-GTGCGCATACCGTCGCCGAC-3'	852	F 5'-ATCCCGGCCGATCACATGAT-3' sR 5'-CTCCAGCAGCCCCTCTTTCAG-3'
pure	F 5'-ATGTCTTCCCGCAATAATCC-3' R 5'-TCATAGCGTCCCCCGCGGATC-3' R1 5'-CGAGAACGCAAACTTGCTTC-3'	510	sF 5'-CGCATTATTCCGGCGCGTGT-3' sF1 5'-CGCAATAATCCGGCGCGTGT-3' sR 5'-CGCGGATCGGGATTTTCCAG-3' sR1 5'-GAACGCAAACTTGCTTCAT-3'
sucA	F 5'-AGCACCGAAGAGAAACGCTG-3' R 5'-GGTTGTTGATAACGATACGTAC-3'	643	sF 5'-AGCACCGAAGAGAAACGCTG-3' sR 5'-GGTTGTTGATAACGATACGTAC-3'
hisD	F 5'-GAAACGTTCCATTCCGCGCAGAC-3' R 5'-CTGAACGGTCATCCGTTTCTG-3'	894	sF 5'-GTCGGTCTGTATATTCCCGG-3' sR 5'-GGTAATCGCATCCACCAAATC-3'
aroC	F 5'-CCTGGCACCTCGCGCTATAC-3' R 5'-CCACACACGGATCGTGGCG-3'	826	sF 5'-GGCACCAGTATTGGCCTGCT-3' sR 5'-CATATGCGCCACAATGTGTTG-3'
hemD	F 5'-ATGAGTATTCTGATCACCCG-3' F1 5'-GAAGCGTTAGTGAGCCGTCTGCG-3' R 5'-ATCAGCGACCTTAATATCTTGCCA-3'	666	sF 5'-GTGGCCTGGAGTTTTCCACT-3' sF1 5'-ATTCTGATCACCCGCCCCTC-3' sR 5'-GACCAATAGCCGACAGCGTAG-3'
dnaN	F 5'-ATGAAATTTACCGTTGAACGTGA-3' R 5'-AATTTCTCATTCGAGAGGATTGC-3' R1 5'-CCGCGGAATTTCTCATTCGAG-3'	833	sF 5'-CCGATTCTCGGTAACCTGCT-3' sR 5'-CCATCCACCAGCTTCGAGGT-3'

[1]Expected product length

REFERENCES

REFERENCES

Achtman, M., et al., 2012. Multilocus Sequence Typing as a Replacement for Serotyping in Salmonella enteric. *PLoS Pathogens,* 8(6), p.e1002776.

Adhikari, B. et al., 2010. Multilocus variable-number tandem-repeat analysis and plasmid profiling to study the occurrence of blaCMY-2 within a pulsed-field gel electrophoresis-defined clade of Salmonella *enterica* serovar Typhimurium. *Applied and Environmental Microbiology,* 76(1), pp.69-74.

Adhikari, B. et al., 2009a. Introduction of new multidrug-resistant *Salmonella enterica* strains into commercial dairy herds. *Journal of Dairy Science,* 92(9), pp.4218-28.

Adhikari, B. et al., 2009b. The role of animal movement, including off-farm rearing of heifers, in the interherd transmission of multidrug-resistant Salmonella. *Journal of dairy science,* 92(9), pp.4229-38.

Alali, W.Q. et al., 2008. Longitudinal study of antimicrobial resistance among *Escherichia coli* isolates from integrated multisite cohorts of humans and swine. *Applied and environmental microbiology,* 74(12), pp.3672-81.

Alcaine, S.D. et al., 2005. Ceftiofur-Resistant *Salmonella* strains Isolated from dairy farms represent multiple widely distributed subtypes that evolved by independent horizontal gene transfer. *Applied and Environmental Microbiology,* 49(10), pp.4061-4067.

Alcaine, S.D. et al., 2006. Multilocus sequence typing supports the hypothesis that cow- and human-associated Salmonella isolates represent distinct and overlapping populations. *Applied and Environmental Microbiology,* 72(12), pp.7575-85.

Alcaine, S.D., Warnick, L.D., & Wiedmann, M., 2007. Antimicrobial resistance in nontyphoidal Salmonella. *Journal of Food Protection,* 70(3), pp.780-90.

Anderson, R. et al., 1997. Case-control study of an outbreak of clinical disease attributable to Salmonella menhaden infection in eight dairy herds. *Journal of the American Veterinary Medical Association,* 210(4), pp.528-530.

Arthur, T.M. et al., 2008. Prevalence and characterization of Salmonella in bovine lymph nodes potentially destined for use in ground beef. *Journal of Food Protection,* 71(8), pp.1685-8.

Barham, A. et al., 2002. Effects of the transportation of beef cattle from the feedyard to the packing plant on prevalence levels of *Escherichia coli* O157 and *Salmonella* spp. *Journal of Food Protection,* 65(2), pp.280-3.

Barrett, T.J., Gerner-Smidt, P. & Swaminathan, B., 2006. Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigations and surveillance. *Foodborne Pathogens and Disease,* 3(1), pp.20-31.

Beach, J.C., Murano, E.A. & Acuff, G.R., 2002. Prevalence of *Salmonella* and *Campylobacter* in beef cattle from transport to slaughter. *Journal of Food Protection*, 65(11), pp.1687-1693.

Bender, J.B. & Shulman, S.A., 2004. Reports of zoonotic disease outbreaks associated with animal exhibits and availability of recommendations for preventing zoonotic disease transmission from animals to people in such settings. *Journal of the American Veterinary Medical Association*, 224(7).

Bender, J.B. et al., 1997. Animal by-products contaminated with salmonella in the diets of lactating dairy cows. *Journal of Dairy Science*, 80(11), pp.3064-3067.

Berge, A.C., Atwill, E., & Sischo, W.M., 2003. Assessing antibiotic resistance in fecal Escherichia coli in young calves using cluster analysis techniques. *Preventive Veterinary Medicine*, 61(2), pp.91-102.

Berge, A.C., Adaska, J. & Sischo, W., 2004. Use of antibiotic susceptibility patterns and pulsed-field gel electrophoresis to compare historic and contemporary isolates of multi-drug-resistant Salmonella *enterica* subsp. *enterica* Serovar Newport. *Applied and Environmental Microbiology*, 70(1), pp.318-323.

Berge, A.C. et al., 2010. Geographic, farm, and animal factors associated with multiple antimicrobial resistance in fecal Escherichia coli isolates from cattle in the western United States. *Journal of the American Veterinary Medical Association*, 236(12), pp.1338-44.

Berge, A.C., Moore, D. & Sischo, W.M., 2006. Prevalence and antimicrobial resistance patterns of Salmonella *enterica* in preweaned calves from dairies and calf ranches. *American Journal of Veterinary Research*, 67(9), pp.1580-8.

Beutlich, J. et al., 2011. Antimicrobial resistance and virulence determinants in European Salmonella genomic island 1-positive Salmonella *enterica* isolates from different origins. *Applied and Environmental Microbiology*, 77(16), pp.5655-5664.

Blau, D.M. et al., 2005. Salmonella in dairy operations in the United States: prevalence and antimicrobial drug susceptibility. *Journal of Food Protection*, 68(4), pp.696-702.

Boxrud, D., 2010. Advances in subtyping methods of foodborne disease pathogens. *Current Opinion in Biotechnology*, 21(2), pp.137-41.

Brenner, F.W. et al., 2000. Salmonella Nomenclature GUEST COMMENTARY. *Journal of Clinical Microbiology*, 38(7), pp. 2465-67.

Brichta-Harhay, D.M. et al., 2011. Diversity of multidrug-resistant *Salmonella enterica* strains associated with cattle at harvest in the United States. *Applied and Environmental Microbiology*, 77(5), pp.1783-96.

Butaye, P. et al., 2006. The clonal spread of multidrug-resistant non-typhi *Salmonella* serotypes. *Microbes and infection*, 8(7), pp.1891-7.

Bäumler, A.J., Hargis, B.M. & Tsolis, R.M., 2000. Tracing the Origins of *Salmonella* Outbreaks. *Nature*, 287(5450), pp.50-52.

CDC, 2012a. Centers for Disease Control and Prevention: *Salmonella* Outbreaks. Available at: http://www.cdc.gov/salmonella/outbreaks.html [Accessed May 3, 2012].

CDC, 2011a. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet Surveillance Report for 2009 (Final Report), Atlanta, Georgia: U.S. Department of Health and Human Services, CDC

CDC, 2012b. HealthyPeople.gov: 2020 Topics & Objectives. Available at: http://www.healthypeople.gov/2020/topicsobjectives2020/objectiveslist.aspx?topicId=14 [Accessed May 2, 2012].

CDC, 2012c. National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Final Report, 2010, Atlanta, Georgia: U.S. Department of Health and Human Services, CDC.

CDC, 2012d. *National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Final Report, 2010*, Atlanta, Georgia.

CDC, 2002. Outbreak of Multidrug-Resistant Salmonella Newport - United States, January-April 2002. *Morbidity and Mortality Weekly Report*, 51(25), pp.545-548.

CDC, 2011b. Surveillance for Foodborne Disease Outbreaks - United States, 2008. *Morbidity and mortality weekly report*, 60(35), pp.1197-1202.

CDC, 2011c. Vital Signs: Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food — Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 1996–2010. *Morbidity and mortality weekly report*, 60(22), pp.749-755.

CLSI, 2010. Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement, Wayne, PA: Clinical Laboratory Standards Institute.

Callaway, T.R. et al., 2005. Fecal prevalence and diversity of Salmonella species in lactating dairy cattle in four states. *Journal of Dairy Science*, 88(10), pp.3603-8.

Carlson, J.C. et al., 2011. Efficacy of European starling control to reduce *Salmonella enterica* contamination in a concentrated animal feeding operation in the Texas panhandle. *BMC Veterinary Research*, 7(1)

Cernicchiaro, N. et al., 2012. Association of wild bird density and farm management factors with the prevalence of E. coli O157 in dairy herds in Ohio (2007-2009). *Zoonoses and Public Health*, pp.1-10.

Chen, S. et al., 2004. Characterization of *Salmonella* serovars isolated from retail meats. *Applied and Environmental Microbiology*, 70(1), pp.1-7.

Cho, S. et al., 2007. Multiple-locus variable-number tandem repeat analysis of *Salmonella* Enteritidis isolates from human and non-human sources using a single multiplex PCR. *FEMS microbiology letters*, 275(1), pp.16-23.

Cobbold R.N. et al., 2006. Long-term persistence of multi-drug-resistant Salmonella *enterica* serovar Newport in two dairy herds. *Journal of the American Veterinary Medical Association*, 228(4) pp. 585-591

Cody, S.H., 1999. Two outbreaks of multidrug-resistant Salmonella serotype Typhimurium DT104 infections linked to raw-milk cheese in Northern California. *The Journal of the American Medical Association*, 281(19), pp.1805-1810.

Cummings, K.J. et al., 2009a. The duration of fecal Salmonella shedding following clinical disease among dairy cattle in the northeastern USA. *Preventive Veterinary Medicine*, 92(1-2), pp.134-9.

Cummings, K.J. et al., 2009b. The incidence of salmonellosis among dairy herds in the northeastern United States. *Journal of Dairy Science*, 92(8), pp.3766-74.

Cummings, K.J. et al., 2010. Salmonella *enterica* serotype Cerro among dairy cattle in New York: an emerging pathogen? *Foodborne pathogens and disease*, 7(6), pp.659-65.

Daniels, J.B. et al., 2009. Role of ceftiofur in selection and dissemination of blaCMY-2-mediated cephalosporin resistance in Salmonella *enterica* and commensal Escherichia coli isolates from cattle. *Applied and Environmental Microbiology*, 75(11), pp.3648-55.

Dargatz, D.A. et al., 2005. Characterization of Escherichia coli and Salmonella *enterica* from cattle feed ingredients. *Foodborne Pathogens and Disease*, 2(4), pp.341-7.

Davis, M.A., 2003. Feedstuffs as a vehicle of cattle exposure to Escherichia coli O157:H7 and Salmonella *enterica*. *Veterinary Microbiology*, 95(3), pp.199-210.

Davis, M.A. et al., 1999. Changes in antimicrobial resistance among Salmonella *enterica* serovar Typhimurium isolates from humans and cattle in the Northwestern United States, 1982-1997. *Emerging Infectious Diseases*, 5(6), pp.802-6.

Davis, M.A., Hancock, D. & Besser, T, 2002. Multiresistant clones of *Salmonella*: The importance of dissemination. *Journal of Laboratory and Clinical Medicine*, 140(3), pp.135-141.

Davis, M.A. et al., 2003. Evaluation of pulsed-field gel electrophoresis as a tool for determining the degree of genetic relatedness between strains of Escherichia coli O157: H7. *Journal of clinical microbiology*, 41(5), pp.1843-1849.

Davis, M.A., Besser, T.E., et al., 2007. Multidrug-resistant Salmonella Typhimurium, Pacific Northwest, United States. *Emerging Infectious Diseases*, 13(10), pp.1583-1586.

Davis, M.A., Hancock, D. et al., 2007. Antimicrobial resistance in Salmonella *enterica* serovar Dublin isolates from beef and dairy sources. *Veterinary microbiology*, 119(2-4), pp.221-30.

Dechet, A.M. et al., 2006. Outbreak of multidrug-resistant Salmonella *enterica* serotype Typhimurium Definitive Type 104 infection linked to commercial ground beef, Northeastern United States, 2003-2004. *Clinical Infectious Diseases*, 42(6), pp.747-52.

Didelot, X. et al., 2011. Recombination and population structure in Salmonella *enterica*. *PLoS genetics*, 7(7), p.e1002191.

Doublet, B. et al., 2005. The Salmonella genomic island 1 is an integrative mobilizable element. *Molecular Microbiology*, 55(6), pp.1911-1924.

Duijkeren, E.V. et al., 2002. Serotype and phage type distribution of Salmonella strains isolated from humans , cattle , pigs , and chickens in The Netherlands from 1984 to 2001. *Journal of Clinical Microbiology*, 40(11), pp.3980-3985.

Edrington, T.S. et al., 2004. Antimicrobial resistance and serotype prevalence of Salmonella isolated from dairy cattle in the southwestern United States. *Microbial Drug Resistance*, 10(1), pp.51-6.

Edrington, T.S. et al., 2008. Prevalence of multidrug-resistant Salmonella on commercial dairies utilizing a single heifer raising facility. *Journal of Food Protection*, 71(1), pp.27-34.

Enne, V.I. et al., 2004. Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. *The Journal of Antimicrobial Chemotherapy*, 53(6), pp.958-63.

FDA, 2012a. Cephalosporin order of prohibition of ELDU. 21 CFR Part 530. Rockville, MD: Department of Health and Human Services: FDA

FDA, 2012b. National Antimicrobial Resistance Monitoring System, Retail Meat Report, 2010. Rockville, Md: Department of Health and Human Services, FDA.

FDA, 2010. National Antimicrobial Resistance Monitoring System - Enteric Bacteria (NARMS): 2007 Executive Report, Rockville, Md: Department of Health and Human Services, FDA.

FDA, 2011. National Antimicrobial Resistance Monitoring System – Enteric Bacteria (NARMS): 2009 Executive Report., Rockville, Md. Department of Health and Human Services, FDA.

Fakhr, M.K., Nolan, L.K. & Logue, C.M., 2005. Multilocus Sequence Typing Lacks the Discriminatory Ability of Pulsed-Field Gel Electrophoresis for Typing Salmonella *enterica* Serovar Typhimurium. *Journal of Clinical Microbiology*, 43(5), pp.2215-2219.

Fey, P.D. et al., 2000. Ceftriaxone-resistant salmonella infection acquired by a child from cattle. *The New England Journal of Medicine*, 342(17), pp.1242-9.

Fitzgerald, A.C. et al., 2003. Antimicrobial susceptibility and factors affecting the shedding of E. coli O157:H7 and Salmonella in dairy cattle. *Letters in Applied Microbiology*, 37(5), pp.392-398.

Foley, S.L. & Lynne, A.M., 2008. Food animal-associated Salmonella challenges: pathogenicity and antimicrobial resistance. *Journal of Animal Science*, 86(14 Suppl), pp.E173-87.

Foley, S.L. et al., 2006. Comparison of subtyping methods for differentiating Salmonella *enterica* serovar Typhimurium isolates obtained from food animal sources. *Journal of Clinical Microbiology*, 44(10), pp.3569-77.

Foley, S.L. et al. 2007. Review: Comparison of Molecular Typing Methods for the Differentiation of Salmonella Foodborne Pathogens. *Foodborne Pathogens and Disease*, 4(3), pp. 253-76.

Fossler, C.P., et al., 2005a. Herd-level factors associated with isolation of Salmonella in a multi-state study of conventional and organic dairy farms I. Salmonella shedding in cows. *Preventive veterinary medicine*, 70(3-4), pp.257-77.

Fossler, C.P. et al., 2005b. Herd-level factors associated with isolation of Salmonella in a multi-state study of conventional and organic dairy farms II. Salmonella shedding in calves. *Preventive veterinary medicine*, 70(3-4), pp.279-91.

Fossler, C.P. et al., 2005c. Cattle and environmental sample-level factors associated with the presence of Salmonella in a multi-state study of conventional and organic dairy farms. *Preventive veterinary medicine*, 67(1), pp.39-53.

Fossler, C.P. et al., 2004. Prevalence of Salmonella spp on conventional and organic dairy farms. *Journal of the American Veterinary Medical Association*, 225(4), pp.567-73.

Fricke, W.F. et al., 2009. Antimicrobial resistance-conferring plasmids with similarity to virulence plasmids from avian pathogenic Escherichia coli strains in Salmonella *enterica* serovar Kentucky isolates from poultry. *Applied and Environmental Microbiology*, 75(18), pp.5963-71.

Frye, J.G. et al., 2008. Analysis of Salmonella *enterica* with reduced susceptibility to the third-generation cephalosporin ceftriaxone isolated from U.S. cattle during 2000-2004. *Microbial Drug Resistance*, 14(4), pp.251-8.

Frye, J.G. et al., 2010. Development of a DNA microarray to detect antimicrobial resistance genes identified in the National Center for Biotechnology Information database. *Microbial Drug Resistance*, 16(1), pp.9-19.

Galanis, E. et al., 2006. Web-based surveillance and global Salmonella distribution, 2000-2002. *Emerging Infectious Diseases*, 12(3), pp.381-8.

Galland, J.C. et al., 2001. Diversity of Salmonella serotypes in cull (market) dairy cows at slaughter. *Journal of the American Veterinary Medical Association*, 219(9), pp.1216-20.

Gardner, I.A., Willeberg, P. & Mousing, J., 2007. Empirical and theoretical evidence for herd size as a risk factor for swine diseases. *Animal Health Research Reviews*, 3(1), pp.43-55.

Gaukler, S.M. et al., 2009. *Escherichia coli* , Salmonella , and Mycobacterium avium subsp . paratuberculosis in Wild European Starlings at a Kansas Cattle Feedlot. *Avian Diseases*, 53(4), pp.544-551.

Gay, J. & Hunsaker, M., 1993. Isolation of multiple Salmonella serovars from a dairy two years after a clinical salmonellosis outbreak. *Journal of the American Veterinary Medical Association*, 203(9), p.1314.

Gebreyes, W.A. et al., 2009. Occurrence of spvA virulence gene and clinical significance for multidrug-resistant Salmonella strains. *Journal of clinical microbiology*, 47(3), pp.777-80.

Giles, N., Hopper, S.A. & Wray, C., 1989. Persistence of S. typhimurium in a large dairy herd. *Epidemiology and infection*, 103(2), pp.235–241.

Glickman, L. et al., 1981. Bovine salmonellosis attributed to Salmonella anatum-contaminated haylage and dietary stress. *Journal of the American Veterinary Medical Association*, 178(12), pp.1268-1272.

Glynn, M.K., Bopp, C., et al., 1998. Emergence of multidrug-resistant Salmonella *enterica* serotype typhimurium DT104 infections in the United States. *The New England Journal of Medicine*, 338(19), pp.1333-1338.

Guerra, B et al., 2000. Molecular characterisation of emergent multiresistant Salmonella *enterica* serotype [4,5,12:i:-] organisms causing human salmonellosis. *FEMS Microbiology Letters*, 190(2), pp.341-7.

Gupta, A. et al., 2003. Emergence of multidrug-resistant Salmonella *enterica* serotype Newport infections resistant to expanded-spectrum cephalosporins in the United States. *The Journal of Infectious Diseases*, 188(11), pp.1707-16.

Habing, G.G. et al. 2012. Farm-Level Associations with the Shedding of Salmonella and Antimicrobial Resistant Salmonella on United States Dairy Herds. *Foodborne Pathogens and Diseases*, In Press.

Habing, G.G., et al. 2012. Changes in the antimicrobial resistance profiles of Salmonella isolated from the same Michigan dairy farms in 2000 and 2009. *Food Research International*, 45(2), pp.919-924.

Hald, T. et al., 2007. The Attribution of Human Infections with Antimicrobial Resistant Salmonella Bacteria in Denmark to Sources of Animal Origin. *Foodborne Pathogens And Disease*, 4(3) pp. 313-24.

Hall, M.A. et al., 1996. Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant Enterobacteriaceae in a clinical setting. *European Journal of Clinical Microbiology & Infectious Diseases*, 17, pp.49-56.

Harbottle, H. et al., 2006. Comparison of multilocus sequence typing, pulsed-field gel electrophoresis, and antimicrobial susceptibility typing for characterization of Salmonella *enterica* serotype Newport isolates. *Journal of Clinical Microbiology*, 44(7), pp.2449-57.

Heithoff, D.M. et al., 2012. Intraspecies variation in the emergence of hyperinfectious bacterial strains in nature. *PLoS Pathogens*, 8(4), p.e1002647.

Helms, M., Simonsen, J. & Molbak, Kare, 2004. Quinolone resistance is associated with increased risk of invasive illness or death during infection with Salmonella serotype Typhimurium. *The Journal of Infectious Diseases*, 190(9), pp.1652-4.

Himathongkham, S. et al., 1999. Survival of Escherichia coli O157: H7 and Salmonella Typhimurium in cow manure and cow manure slurry. *FEMS Microbiology Letters*, 178. pp. 251-57

Hoelzer, K. et al., 2011. Salmonella Cerro isolated over the past twenty years from various sources in the US represent a single predominant pulsed-field gel electrophoresis type. *Veterinary Microbiology*, pp.1-5.

Hoelzer, K. et al., 2010. The prevalence of multidrug resistance is higher among bovine than human Salmonella *enterica* serotype Newport, Typhimurium, and 4,5,12:i:- Isolates in the United States but differs by serotype and geographic region. *Applied and Environmental Microbiology*, 76(17), pp.5947-59.

Hume, M.E. et al., 2004. Salmonella genotype diversity in nonlactating and lactating dairy cows. *Journal of Food Protection*, 67(10), pp.2280-3.

Humphrey, T. & Cruickshank, J., 1985. Antibiotic and deoxycholate resistance in Campylobacter jejuni following freezing or heating. *Journal of Applied Bacteriology*, 59, pp.65-71.

Hunter, P.R. & Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of clinical microbiology*, 26(11), pp.2465–2466.

Huston, C.L. et al., 2002. Prevalence of fecal shedding of Salmonella spp in dairy herds. *Journal of the American Veterinary Medical Association*, 220(5), pp.645-9.

Ibarra, J.A. & Steele-Mortimer, O., 2009. Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival. *Cellular microbiology*, 11(11), pp.1579-86.

Islam, M. et al., 2004. Persistence of Salmonella *enterica* Serovar Typhimurium on Lettuce and Parsley and in Soils on Which They Were Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water. *Foodborne Pathogens And Disease*, 1(1), pp.27-35.

Jones, P.W. et al., 1982. Transmission of Salmonella Mbandaka to cattle from contaminated feed. *Journal of Hygiene*, 88(2), pp.255-263.

Kabagambe, E.K. et al., 2000. Risk factors for fecal shedding of Salmonella in 91 US dairy herds in 1996. *Preventive Veterinary Medicine*, 43(3), pp.177-94.

Kaneene, J.B. et al., 2007. Considerations when using discriminant function analysis of antimicrobial resistance profiles to identify sources of fecal contamination of surface water in Michigan. *Applied and Environmental Microbiology*, 73(9), pp.2878-90.

Kaneene, J.B. et al., 2009. Changes in multidrug resistance of enteric bacteria following an intervention to reduce antimicrobial resistance in dairy calves. *Journal of Clinical Microbiology*, 47(12), pp.4109-12.

Kaneene, J.B. et al., 2008. Changes in tetracycline susceptibility of enteric bacteria following switching to nonmedicated milk replacer for dairy calves. *Journal of Clinical Microbiology*, 46(6), pp.1968-77.

Kauffman, F., 1975. *Classification of bacteria: A realistic scheme with special reference to the classification of Salmonella- and Escherichia-species*, Copenhagen: Munksgaard.

Van Kessel, J.S. et al., 2007. Longitudinal study of a clonal, subclinical outbreak of Salmonella *enterica* subsp. *enterica* serovar Cerro in a US dairy herd. *Foodborne pathogens and disease*, 4(4), pp.449–461.

Van Kessel, J.S. et al., 2012. Dynamics of Salmonella serotype shifts in an endemically infected dairy herd. *Foodborne Pathogens and Disease*, 9(4), pp.1-6.

Khachatryan, A.R. et al., 2006. Use of a nonmedicated dietary supplement correlates with increased prevalence of streptomycin-sulfa-tetracycline-resistant Escherichia coli on a dairy farm. *Applied and Environmental Microbiology*, 72(7), pp.4583-8.

Kidd, R., Rossignol, A. & Gamroth, M., 2002. Salmonella and other Enterobacteriaceae in dairy-cow feed ingredients: antimicrobial resistance in western Oregon. *Journal of Environmental Health*, 64, pp.9-16.

Kotetishvili, M. et al., 2002. Multilocus Sequence Typing for Characterization of Clinical and Environmental Salmonella Strains. *Journal of Clinical Microbiology*, 40(5), pp.1626-1635.

Krauland, M.G. et al., 2009. Integron-mediated multidrug resistance in a global collection of nontyphoidal Salmonella *enterica* isolates. *Emerging Infectious Diseases*, 15(3), pp.388-96.

Kunze, D.J. et al., 2008. Salmonella *enterica* burden in harvest-ready cattle populations from the southern high plains of the United States. *Applied and Environmental Microbiology*, 74(2), pp.345-51.

Lan, R., Reeves, P.R. & Octavia, S., 2009. Population structure, origins and evolution of major Salmonella *enterica* clones. *Infection, genetics and evolution*, 9(5), pp.996-1005.

LeJeune, J.T. & Christie, N.P., 2004. Microbiological quality of ground beef from conventionally-reared cattle and "raised without antibiotics" label claims. *Journal of Food Protection*, 67(7), pp.1433-7.

Lindqvist, N. et al., 1999. Discrimination between endemic and feedborne Salmonella Infantis infection in cattle by molecular typing. *Epidemiology and Infection*, 122, pp.497-504.

Litrup, E. et al., 2010. Association between phylogeny, virulence potential and serovars of Salmonella *enterica*. *Infection, genetics and evolution*, 10(7), pp.1132-1139.

Liu, W.B. et al., 2011. Diversity of Salmonella isolates using serotyping and multilocus sequence typing. *Food microbiology*, 28(6), pp.1182-9.

Losinger, W.C. et al., 1995. Management factors related to Salmonella shedding by dairy heifers. *Journal of Dairy Science*, 78(11), pp.2464-2472.

Lynch, M., Painter, J. & Woodruff, R., 2006. Surveillance for Foodborne: Disease Outbreaks: United States, 1998-2002. *Morbidity and Mortality Weekly Report*, 55(SS-10).

Majowicz, S.E. et al., 2010. The global burden of nontyphoidal Salmonella gastroenteritis. *Clinical infectious diseases*, 50(6), pp.882-9.

Maragakis, L.L., Perencevich, E.N. & Cosgrove, S.E., 2008. Clinical and economic burden of antimicrobial resistance. *Expert review of anti-infective therapy*, 6(5), pp.751-63.

Martin, L.J. et al., 2004. Increased burden of illness associated with antimicrobial-resistant Salmonella *enterica* serotype typhimurium infections. *The Journal of Infectious Diseases*, 189(3), pp.377-84.

Martiny, J.B.H. et al., 2006. Microbial biogeography: putting microorganisms on the map. *Nature reviews. Microbiology*, 4(2), pp.102-12.

Mazurek, J. et al., 2004. A multistate outbreak of Salmonella *enterica* serotype Typhimurium infection linked to raw milk consumption--Ohio, 2003. *Journal of Food Protection*, 67(10), pp.2165-70.

McDonough, P L et al., 1999. Salmonella *enterica* serotype Dublin infection: an emerging infectious disease for the northeastern United States. *Journal of Clinical Microbiology*, 37(8), pp.2418-27.

McLaren, I. & Wray, C., 1991. Epidemiology of Salmonella Typhimurium infection in calves: persistence of salmonellae on calf units. *Veterinary Record*, 129, pp.461-462.

Mead, P. et al., 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases*, 5(6), pp.840-2.

Michael, Geovana Brenner et al., 2006. Genes and mutations conferring antimicrobial resistance in Salmonella: an update. *Microbes and infection*, 8(7), pp.1898-914.

Mollenkopf, D., Kleinhenz, K. & Funk, J., 2011. Salmonella *enterica* and Escherichia coli Harboring bla-CMY in Retail Beef and Pork Products. *Foodborne Pathogens and Diseases*, 8(2). pp. 333-36

Nicholson, F.A., Groves, S.J. & Chambers, B.J., 2005. Pathogen survival during livestock manure storage and following land application. *Bioresource technology*, 96(2), pp.135-43.

Oliver, S.P. et al., 2009. Food safety hazards associated with consumption of raw milk. *Foodborne Pathogens and Disease*, 6(7), pp.793-806.

Oppegaard, H., Steinum, T.M. & Wasteson, Y., 2001. Horizontal transfer of a multi-drug resistance plasmid between coliform bacteria of human and bovine origin in a farm environment. *Applied and Environmental Microbiology*, 67(8), p.3732.

Paterson, D L et al., 2001. Outcome of cephalosporin treatment for serious infections due to apparently susceptible organisms producing extended-spectrum beta-lactamases: implications for the clinical microbiology laboratory. *Journal of Clinical Microbiology*, 39(6), pp.2206-12.

Pavia, A.T. et al., 1990. Epidemiologic Evidence that Prior Antimicrobial Exposure Decreases Resistance to Infection by Antimicrobial-Sensitive Salmonella. *Journal of Infectious Diseases*, 161, pp.255-260.

Pedersen, T.B., Olsen, J.E. & Bisgaard, M., 2008. Persistence of Salmonella Senftenberg in poultry production environments and investigation of its resistance to desiccation. *Avian pathology*, 37(4), pp.421-7.

Peek, S.E. et al., 2004. Isolation of Salmonella spp from the environment of dairies without any history of clinical salmonellosis. *Journal of the American Veterinary Medical Association*, 225(4), pp.574-7.

Rabsch, W., Tschäpe, H. & Bäumler, A.J., 2001. Non-typhoidal salmonellosis: emerging problems. *Microbes and Infection*, 3, pp.237-247.

Ray, K.A. et al., 2006. Antimicrobial susceptibility of Salmonella from organic and conventional dairy farms. *Journal of Dairy Science*, 89(6), pp.2038-50.

Ray, K. a et al., 2007. Prevalence of antimicrobial resistance among Salmonella on midwest and northeast USA dairy farms. *Preventive veterinary medicine*, 79(2-4), pp.204-23.

Ribot, E.M. et al., 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella, and Shigella for PulseNet. *Foodborne pathogens and disease*, 3(1), pp.59-67.

Rostagno, M.H., Hurd, H. Scott & McKean, J.D., 2011. Variation of bacteriologic and serologic Salmonella *enterica* prevalence between cohorts within finishing swine production farms. *Food Research International*, 45(2) pp. 867-70

Rotger, R. & Casadesús, J., 1999. The virulence plasmids of Salmonella. *International microbiology*, 2(3), pp.177-84.

Ruzante, J.M. et al., 2010. Factors associated with Salmonella presence in environmental samples and bulk tank milk from US dairies. *Zoonoses and Public Health*, 57(7-8), pp.e217-25.

Ryan, C.A. et al., 1987. Massive Outbreak of Antimicrobial-Resistant Salmonellosis Traced to Pasteurized Milk. *Journal of the American Medical Association*, 258 (22) pp. 3269-74

Rychlik, I., Gregorova, D. & Hradecka, H., 2006. Distribution and function of plasmids in Salmonella *enterica*. *Veterinary Microbiology*, 112(1), pp.1-10.

Sakoulas, G. et al., 2004. Relationship of MIC and bactericidal activity to efficacy of vancomycin for treatment of methicillin-resistant Staphylococcus aureus bacteremia. *Journal of Clinical Microbiology*, 42(6), pp.2398-402.

Sangal, V. et al., 2010. Evolution and population structure of Salmonella *enterica* serovar Newport. *Journal of Bacteriology*, 192(24), pp.6465-76.

Sarwari, A.R., Magder, L.S., Levine, P., McNamara, A.M., Knower, S., Armstrong, G.L., Etzel, R., Hollingsworth, J. & Morris, J G, 2001. Serotype distribution of Salmonella isolates from food animals after slaughter differs from that of isolates found in humans. *The Journal of Infectious Diseases*, 183(8), pp.1295-9.

Sato, K et al., 2004. Comparison of antimicrobial susceptibility of Staphylococcus aureus isolated from bulk tank milk in organic and conventional dairy herds in the midwestern United States and Denmark. *Journal of Food Protection*, 67(6), pp.1104-10.

Scallan, E., 2011. Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases*, 17(1), pp.7-15.

Sickle, J.V., 1997. Using Mean Similarity Dendrograms to Evaluate Classifications. *Journal of Agricultural, Biological, and Environmental Statistics*, 2(4), p.370.

Singer, R.S., Sischo, W.M. & Carpenter, T.E., 2004. Exploration of Biases That Affect the Interpretation of Restriction Fragment Patterns Produced by Pulsed-Field Gel Electrophoresis. *Journal of Clinical Microbiology*, 42(12), pp.5502-5511.

Soyer, Y. et al., 2010. Pulsed-field gel electrophoresis diversity of human and bovine clinical Salmonella isolates. *Foodborne Pathogens and Disease*, 7(6), pp.707-17.

Spika, J. et al., 1987. Chloramphenicol-resistant Salmonella Newport traced through hamburger to dairy farms: a major persisting source of human salmonellosis in California. *New England Journal of Medicine*, 316(10), pp.565-570.

Wittum T.E. et al., 2010. CTX-M-Type Extended-Spectrum B-lactamses present in *Escherichia coli* from the feces of cattle in Ohio, United States. *Foodborne Pathogens And Disease*, 7(12) pp.1575-1579.

Steele-Mortimer, O., 2008. The Salmonella-containing vacuole: moving with the times. *Current Opinion in Microbiology*, 11(1), pp.38-45.

Stepan, R.M. et al., 2011. Molecular and comparative analysis of Salmonella *enterica* Senftenberg from humans and animals using PFGE, MLST and NARMS. *BMC Microbiology*, 11(1), p.153.

Stevens, M.P., Humphrey, T.J. & Maskell, D.J., 2009. Molecular insights into farm animal and zoonotic Salmonella infections. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 364(1530), pp.2709-23.

Sukhnanand, S. et al., 2005. DNA Sequence-Based Subtyping and Evolutionary Analysis of Selected Salmonella *enterica* Serotypes. *Journal of Clinical Microbiology*, 43(8) pp.3688-98.

Swaminathan, B et al., 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerging Infectious Diseases*, 7(3), pp.382-9.

Tacket, C.O. et al., 1985. An outbreak of multiple-drug-resistant Salmonella enteritis from raw milk. *The journal of the American Medical Association*, 253(14), pp.2058-60.

Tappero, J.W. et al., 1993. Reduction in the incidence of human listeriosis in the United States: effectiveness of prevention efforts? *The journal of the American Medical Association*, 273 (14) pp. 1118-22.

Tenover, F.C., 2006. Mechanisms of antimicrobial resistance in bacteria. *American Journal of Infection Control*, 34(5 Suppl 1), pp.S3-10; discussion S64-73.

Tenover, F.C. et al., 1995. Guest Commentary: interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology*, 33(9), pp.2233-2239.

Threlfall, E.J. et al., 2006. Assessment of factors contributing to changes in the incidence of antimicrobial drug resistance in Salmonella *enterica* serotypes Enteritidis and Typhimurium from humans in England and Wales in 2000, 2002 and 2004. *International Journal of Antimicrobial Agents*, 28(5), pp.389-95.

Threlfall, E.J., Hopkins, K. & Ward, L., 2005. Diversification in Salmonella Typhimurium DT104. *Emerging Infectious Diseases*, 11(6), pp.980-981.

Threlfall, E.J., 2000. Epidemic Salmonella typhimurium DT104 — a truly international multiresistant clone. *Journal of Antimicrobial Chemotherapy*, 46, pp.7-10.

Torpdahl, M. et al., 2005. Genotypic characterization of Salmonella by multilocus sequence typing, pulsed-field gel electrophoresis and amplified fragment length polymorphism. *Journal of Microbiological Methods*, 63(2), pp.173-84.

Toth, J D et al., 2011. Survival characteristics of Salmonella *enterica* serovar Newport in the dairy farm environment. *Journal of Dairy Science*, 94(10), pp.5238-46.

Tragesser, L.A. et al., 2006. Association between ceftiofur use and isolation of Escherichia coli with reduced susceptibility to ceftriaxone from fecal samples of dairy cows. *American journal of Veterinary Research*, 67(10), pp.1696-700.

USDA, 2011a. *Clostridium difficile* on U. S. Dairy Operations, Fort Collins, CO: USDA:APHIS:VS, CEAH.

USDA, 2009. Dairy 2007, Part IV: Reference of Dairy Cattle Health and Management Practices in the United States, 2007. USDA:APHIS:VS, CEAH.

USDA, 2012. National Antimicrobial Resistance Monitoring System Animal Isolates: Table 1. Percent Resistance in Salmonella Isolates from Diagnostic Dairy Cattle. Available at: http://www.ars.usda.gov/SP2UserFiles/Place/66120508/NARMS/percent_resistance/SalmDairyCattleDiag.pdf [Accessed June 09, 2012].

USDA, 2012a. National Antimicrobial Resistance Monitoring System – Enteric Bacteria, Animal Arm (NARMS): 2010 NARMS Animal Arm Annual Report, Athens, GA: USDA, Agricultural Research Service.

USDA, 2008. National Antimicrobial Resistance Monitoring System: Enteric Bacteria. Veterinary Isolates Final Report - Slaughter Isolates. Athens, GA: USDA, Agricultural Research Service.

USDA, 2011b. *Salmonella, Listeria,* and *Campylobacter* on U.S. Dairy Operations, 1996-2007, Fort Collins, CO: USDA:APHIS:VS, CEAH.

USDA, 2012b. Serotype profile of salmonella isolates from meat and poultry products: January 1998 through December 2010. USDA: Food Safety and Inspection Service.

Vanselow, B.A. et al., 2007. Salmonella Typhimurium persistence in a Hunter Valley dairy herd. *Australian Veterinary Journal*, 85(11), pp.446-50.

Varma, J.K. et al., 2005a. Antimicrobial-resistant nontyphoidal Salmonella is associated with excess bloodstream infections and hospitalizations. *The Journal of infectious diseases*, 191(4), pp.554-61.

Varma, J.K. et al., 2005b. Hospitalization and antimicrobial resistance in Salmonella outbreaks, 1984-2002. *Emerging Infectious Diseases*, 11(6), pp.943-945.

Varma, J.K. et al., 2006. Highly Resistant Salmonella Newport-MDR AmpC transmitted through the domestic US food supply: A FoodNet case-control study of sporadic Salmonella Newport infections, 2002 – 2003. *The Journal of Infectious Diseases*, 194, pp.222-30.

Voetsch, A.C. et al., 2004. FoodNet estimate of the burden of illness caused by nontyphoidal Salmonella infections in the United States. *Clinical Infectious Diseases*, 38 Suppl 3(Suppl 3), pp.S127-34.

WHO, 2007. Critically Important Antimicrobials for Human Medicine: Categorization for the Development of Risk Management Strategies to contain Antimicrobial Resistance due to Non-Human Antimicrobial Use, Copenhagen, Denmark: World Health Organization.

Wagner, B., Morley, P. & Dargatz, DA, 2003. Short-term repeatability of measurements of antimicrobial susceptibility of Escherichia coli isolated from feces of feedlot cattle. *Journal of Veterinary Diagnostic Investigation*, 15, pp.535-542.

Walk, S.T. et al., 2007. Influence of antibiotic selection on genetic composition of Escherichia coli populations from conventional and organic dairy farms. *Applied and Environmental Microbiology*, 73(19), pp.5982-9.

Warnick, L.D. et al., 2001. Risk factors for clinical salmonellosis in Virginia, USA cattle herds. *Preventive Veterinary Medicine*, 49(3-4), pp.259-75.

Warnick, L.D et al., 2003. Effect of previous antimicrobial treatment on fecal shedding of Salmonella *enterica* subsp. *enterica* serogroup B in New York dairy herds with recent clinical salmonellosis. *Preventive Veterinary Medicine*, 56(4), pp.285-297.

Wedel, S.D., et al., 2005. Antimicrobial-drug susceptibility of human and animal Salmonella typhimurium, Minnesota, 1997-2003. *Emerging infectious diseases*, 11(12), pp.1899-906.

Wells, S J et al., 2001. Fecal shedding of Salmonella spp. by dairy cows on farm and at cull cow markets. *Journal of Food Protection*, 64(1), pp.3-11.

Wiesner, M. et al., 2009. Association of virulence plasmid and antibiotic resistance determinants with chromosomal multilocus genotypes in Mexican Salmonella *enterica* serovar Typhimurium strains. *BMC Microbiology*, 15, pp.1-15.

Winokur, P.L. et al., 2001. Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. *Journal of Clinical Microbiology*, 45(10), pp.2716-2722.

You, Y. et al., 2006. Survival of Salmonella *enterica* Serovar Newport in manure and manure-amended soils. *Applied and Environmental Microbiology*, 72(9), pp.5777-5783.

Zhang, Q. et al., 2006. Fitness of antimicrobial-resistant *Campylobacter* and Salmonella. *Microbes and infection*, 8(7), pp.1972-8.

Zhao, C. et al., 2001. *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the greater Washington D. C. Area. *Applied and Environmental Microbiology*, 67(12), pp. 5431-5436.

Zhao, C. et al., 2003a. Characterization of *Salmonella enterica* serotype Newport Isolated from humans and food animals. *Journal of Clinical Microbiology*, 41(12), pp.5366-5371.

Zheng, J. et al., 2011. Simultaneous analysis of multiple enzymes increases accuracy of pulsed-field gel electrophoresis in assigning genetic relationships among homogeneous Salmonella strains. *Journal of Clinical Microbiology*, 49(1), pp.85-94.