A Study of a Bacteriophage-Resistant *Salmonella* Mutant and Factors Influencing Prevalence of *Salmonella* in a Multi-Species Animal Facility

by

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A thesis submitted to the Graduate Faculty of Auburn University in partial fulfillment of the requirements for the Degree of Master of Science

Auburn, Alabama December 10, 2016

Keywords: *Salmonella*, Epidemiology, Environmental, Veterinary, Bacteriophage, Bovine

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Abstract

Bacteriophage (phage) treatment for the reduction of multiple drug resistant *Salmonella* Newport in dairy calves is being examined in our lab from a clinical disease and food safety perspective. An unintended consequence of phage treatment could be the emergence of fully virulent but phage resistant *Salmonella*. Working from the hypothesis that resistance would attenuate virulence in *S*. Newport, we generated a spontaneous mutant resistant to 4 out of 5 lytic phages used in our treatment regimen. Two pairs of 8-10 week old calves were challenged orally with the phage resistant mutant, with one pair administered a dose of $7.45 \times 10^9$ colony forming units (CFU’s) and the second pair $1.47 \times 10^{10}$ CFU’s. A third pair of calves was challenged with a total dose of $1.96 \times 10^{10}$ CFU’s composed of a 1:1.3 ratio of parent:mutant in a competition assay designed to determine how well the mutant competed against the parent strain *in vivo*. The four calves inoculated with the phage resistant *S*. Newport mutant strain alone showed much reduced *Salmonella* fecal shedding and no clinical disease signs. The calves in the competition assay showed severe diarrhea and high fecal shedding of both parent and mutant *Salmonella* strains, along with signs of fever and lethargy. The decreased shedding and absence of signs in the calves which received the phage resistant strain alone indicates that the spontaneous generation of multi-phage resistance in *S*. Newport had an attenuating effect on virulence perhaps by decreasing the mutant’s fitness and/or its ability to colonize its host. The results of the competition experiment suggest that the dual inoculation with parent and mutant strain restored virulence to the mutant strain and may increase fitness in the parent strain. Future experiments designed to examine the mechanism of phage resistance in this attenuated *S*. Newport mutant are planned.

Diseases caused by serotypes from the bacterial genus *Salmonella* can have a major impact on animal and human health. Little research has been performed to examine factors contributing to *Salmonella* incidence in multi-species animal production facilities such as veterinary teaching hospitals and the ability of *Salmonella* serovars to move to adjacent facilities.
We hypothesize that *Salmonella* can move between proximally located animal facilities and pastures and that specific factors increase the likelihood of isolating environmental *Salmonella* serotypes in these locations. Over two years, 631 samples were collected from various large animal facilities and pastures within a veterinary school. Data was recorded to assess factors that contribute to increased prevalence of environmental *Salmonella* contamination. Samples were processed for *Salmonella* isolation as described in the USDA FSIS Microbiology Laboratory Guidebook. *Salmonella* isolates were submitted to the NVSL, Ames, IA, or Biovet, Inc., for serotyping. Data was analyzed with Statistical Analysis System (SAS). Of the 631 samples obtained, 230 (36%) samples were positive for at least one *Salmonella* serotype. *Salmonella* was recovered from the majority of facilities and areas sampled regardless of animal species exposure. A Risk Ratio and Fisher’s Exact Test was used for bivariate analysis of factors associated with *Salmonella* isolation. The factors shown to be significant were season, resident species, and environment. Variables associated with the isolation of *Salmonella* from environmental samples included summer season, water and drain samples, samples exposed to bovine residential animals, indoor environments such as buildings, and the dairy and food animal barns locations with isolation of *Salmonella* serovars. The significantly increased frequency of *Salmonella* isolation from environmental samples exposed to dairy cattle indicates that this species is either the source of this pathogen, or is serving as an amplifying host for *Salmonella*. The recovery of cattle-associated serotypes supports this conclusion.
Acknowledgments

Firstly, I would like to express my sincere gratitude to my major professor Dr. Stuart Price for the support, patience, guidance, and immense knowledge with the research and writing of this thesis. I would like to thank the rest of my committee: Dr. James Wright, Dr. Stephanie Ostrowski, and Dr. Paul Walz for their insightful comments, encouragement, and the furtherance of my research. A special thanks goes to Dr. Wright for his tutelage with statistical analysis and direction of an epidemiological investigation. I want to thank Dr. Sue Duran for her overwhelming assistance in the operations of the teaching hospital and the collection of environmental samples. I thank Forrest Shirley and Dr. Joanna Hyland, fellow grad students, for their stimulating discussions and efforts in my research. I would also like to thank Dr. Philippe Gaillard, Cassandra Kitchens, Heather Worley, Austin Conley, Bailey Snavely, Terri Hathcock, Stephanie Wilson, Amélie Rivaleau, Chris Franklin, Jasmine Morris, and various hospital section heads and administrators for their support and contributions to this research.

I dedicate my thesis work to my father, Dennis, who instilled the passion and drive in me to overcome adversity and strive to be the best I can. I would like to thank Sean Bowden for being such a wonderful friend. A thank you goes to Dr. Culbreth, Dr. Carr, and Dr. Watson for believing in me. I would also like to thank my mother, Lorie, and my siblings, David, Michael, and Velora, for providing me with love, support, and continuous encouragement. Finally, I must express my very profound gratitude to my wife and son, Cassandra and Abel. This accomplishment would not have been possible without them and their patience of so many nights of missing me. Thank you.
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## List of Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td><em>Salmonella</em></td>
<td><em>Salmonella enterica</em> spp. <em>enterica</em></td>
</tr>
<tr>
<td><em>Salmonella</em> spp</td>
<td><em>Salmonella</em> species</td>
</tr>
<tr>
<td>Phage</td>
<td>Bacteriophage</td>
</tr>
<tr>
<td>AUCVM</td>
<td>Auburn University College of Veterinary Medicine</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers of Disease Control and Prevention</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>NVSL</td>
<td>National Veterinary Services Laboratories</td>
</tr>
<tr>
<td>WKLM</td>
<td>White-Kauffmann-Le Minor Scheme</td>
</tr>
<tr>
<td>xMAP SSA</td>
<td>xMAP <em>Salmonella</em> serotyping assay</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>ECA</td>
<td>Enterobacterial common antigen</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SPI</td>
<td><em>Salmonella</em> pathogenicity island</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>SM</td>
<td>Salt-magnesium buffer</td>
</tr>
<tr>
<td>TTh</td>
<td>Tetrathionate enrichment broth</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-bertani broth</td>
</tr>
<tr>
<td>LBM</td>
<td>Luria-bertani broth containing 1mM magnesium</td>
</tr>
<tr>
<td>XLT4</td>
<td>Xylose lysine agar supplemented with tergitol 4</td>
</tr>
<tr>
<td>Nal</td>
<td>Nalidixic Acid</td>
</tr>
<tr>
<td>Rif</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>OD&lt;sub&gt;620&lt;/sub&gt;</td>
<td>Absorbance, or optical density, measured at a wavelength of 620 nm</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>°F</td>
<td>Degrees Fahrenheit</td>
</tr>
<tr>
<td>xg</td>
<td>Times gravity</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis System</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole-genome sequencing</td>
</tr>
<tr>
<td>wgMLST</td>
<td>Whole-genome multilocus sequence typing</td>
</tr>
<tr>
<td>ICP</td>
<td>Infection control program</td>
</tr>
<tr>
<td>ORIG SURV</td>
<td>Original environmental surveillance</td>
</tr>
<tr>
<td>MOD SURV</td>
<td>Modified environmental surveillance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>NICU</td>
<td>Neonatal intensive care unit</td>
</tr>
<tr>
<td>ICP</td>
<td>Infection control program</td>
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<tr>
<td>Fig.</td>
<td>Figure</td>
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</table>
Chapter 1. Literature Review

1.1. Salmonella Introduction

The genus *Salmonella*, a member of the *Enterobacteriaceae*, is a Gram-negative facultatively-anaerobic and peritrichously flagellated bacilli. *Salmonella* serovars are distinguished from members of other genera of the *Enterobacteriaceae* by a combination of biochemical reactions such as the production of hydrogen sulfide, ability to metabolize citrate as a sole carbon source, utilize lysine as a nitrogen source, and use of tetrathionate as a terminal electron acceptor (Sterzenbach et al., 2013). The bacterium is named after the American veterinarian Daniel E. Salmon, who first isolated *Bacillus cholera-suis* from a pig suffering from hog cholera (Salmon and Smith, 1885; Smith, 1894). *B. cholera-suis* has since been renamed *Salmonella enterica* subspecies *enterica* serovar Choleraesuis, abbreviated to *S. enterica* serovar Choleraesuis or *S. Choleraesuis*.

1.2. Salmonella Nomenclature

The nomenclature of the genus *Salmonella* undergone several iterations during the past 40 years. There has been a complicated history of classification of salmonellae from many investigators using phenotypic, serologic, and genotypic methods to determine the phylogeny of *Salmonella*. Based on DNA similarity, in 1987 Leon Le Minor and Michel Popoff proposed two species within the *Salmonella* genus, *enterica* and *bongori* (Barrow et al., 2012). The species *S. enterica* has since been divided into six subgenera: subgenera I (*S. enterica* subspecies (subsp.) *enterica*), subgenera II (*S. enterica* subsp. *salamae*), subgenera IIIa (*S. enterica* subsp. *arizonae*), subgenera IIIb (*S. enterica* subsp. *diarizonae*), subgenera IV (*S. enterica* subsp. *houtenae*), and subgenera VI (*S. enterica* subsp. *indica*). *S. enterica* subspecies V was the original designation for *S. bongori*, which has since been determined a separate species (Grimont and Weill, 2007; Liu et al., 2014).

The seven members of the genus *Salmonella* have been further subtyped by serological methods into more than 2500 serovars. Serovars of *S. enterica* are designated by their antigenic formula, for example *S. enterica* subsp. *enterica* serovar Typhimurium may more simply be
designated by the synonyms *S. enterica* Typhimurium or just *S.* Typhimurium. The antigenic formulas of *Salmonella* are derived from the antigenic properties of their lipopolysaccharide (LPS) sugar repeat units (O-antigens) and their flagellar structural protein subunits (H-antigens). The method of deriving antigenic formulas for serovars is called the White-Kauffmann-Le Minor (WKLM) scheme (Grimont and Weill, 2007). As an example, the antigenic formula for *Salmonella* Newport is 6,8,20:e,h :1,2 :[z67],[z78] and *Salmonella* Typhimurium is 1,4,[5],12 :i :1,2. In a few serotypes, *S.* Typhi, *S.* Dublin, and *S.* Paratyphi C, a capsular polysaccharide antigen (Vi-antigen) can be found.

Serotyping has become the accepted standard method to differentiate *Salmonella* serovars, and is an important tool in public health. The limitations of traditional serotyping methods are the expense of antisera, the time-consuming nature of the procedure, the requirement for well-trained technicians, and the fact that some isolates cannot be typed (Abatcha et al., 2014). New systems are aimed at identifying *Salmonella enterica* serovars based on the genes coding for the somatic O and H-antigens. A group at the United States of America (USA) Centers for Disease Control and Prevention (CDC) started systematically sequencing various alleles of the flagellin genes (McQuiston et al., 2004). They identified and determined genetic signatures for 67 of the 114 known flagellar antigenic types. Subsequently, McQuiston et al. (2011) used a DNA bead-based liquid array, and designed specific polymerase chain reaction (PCR) primers and probes for the determination of 36 flagellar antigen genes of *Salmonella* (McQuiston et al., 2011). Also at the CDC, Fitzgerald et al (2007) developed a related strategy for serogroup identification based on the O-antigen rfb genes, from which signature probes were derived and integrated into a suspension bead (Luminex Technology) fluorescence assay (Fitzgerald et al., 2007). Utilizing a combination of serogroup-typing probes and the flagellar-gene-typing system, Luminex Corporation (Austin, TX) developed a bead-based suspension array assay, the xMAP *Salmonella* serotyping assay (xMAP SSA). Diagnostic laboratories, such as Biovet, Inc., utilize the Luminex platform for the xMAP SSA multiplex nucleic acid-based direct hybridization assay to rapidly identify 7 O-antigens, 35 H-antigens, and three additional targets (*sdf* [specific for *S.* Enteritidis], *fljB* [positive control for second H Antigen phase], and the Vi antigen) (Christopher-Hennings et al., 2013; Dunbar et al., 2015).
1.3. *Salmonellosis and Host-Specificity*

Although members of the genus *Salmonella* are genetically closely related, there are wide variations in host-specificity, virulence and disease manifestations. The acquisition or loss of certain genes plays an important role in the evolution of different serovars.

Most *Salmonella* infections in humans and warm blooded animals belong to subspecies I or *S. enterica* species. *S. enterica* serovars are typically transmitted by the fecal-oral route. *Salmonella* pathogens cause four major overlapping syndromes: enteric fever, bacteremia, enterocolitis/diarrhea, and chronic asymptomatic carriage (Coburn et al., 2007). Salmonellosis clinically is seen ranging from no clinical disease to gastroenteritis to septicemia. *Salmonella* serovars are typically described as typhoidal *Salmonella* and non-typhoidal *Salmonella*. Non-typhoidal *Salmonella* serovars such as *S.* Typhimurium generally have a broad host range. These serovars may generate different disease manifestations in different hosts such as asymptomatic carriage, gastroenteritis, and bacteremia or focal systemic infections (Gal-Mor et al., 2014).

*Salmonella* serovars are divided into groups based on their host range: “ubiquitous” (non-adapted, broad host range), “host-adapted”, and “host-restricted” (Evangelopoulou et al., 2013; Uzzau et al., 2000). *Salmonella* serovars with narrow host ranges are described as “host-adapted” and may have the ability to disseminate from the gastrointestinal tract, colonize systemic sites, and persist systemically for long periods of time in their more preferred hosts (Monack, 2012). Typhoidal *Salmonella* serovars which cause enteric fever are typical host-adapted pathogens with a narrow host range (Gal-Mor et al., 2014). “Host-adapted” serovars, such as *S.* Dublin (in cattle) and *S.* Choleraesuis (in pigs) which cause systemic disease and bacteremia in their preferred hosts may accidentally infect other species. These non-preferred hosts typically have subclinical infections. Host factors affect susceptibility to these serovars and affect the disease manifestation as well as the potential for the host to become a carrier or “symptomless excreter”. Such animals can be a health hazard to susceptible animal species due to contamination of the environment of preferred hosts (Evangelopoulou et al., 2013). Serovars such as *S.* Typhi in humans and higher primates, *S.* Gallinarum in chickens, *S.* Abortusovis in sheep, *S.* Typhisuis in pigs, and *S.* Abortusequi in horses are considered “host-restricted” serovars and are highly host adapted, almost exclusively associated with systemic disease or enteric fever in one specific host species (Agbaje et al., 2011; Evangelopoulou et al., 2013; Uzzau et al., 2000).
Salmonella disease manifestations vary depending on the host that is infected. S. Dublin, S. Typhimurium and S. Choleraesuis are capable of causing disease in both humans and animals, but the disease can vary among different hosts. S. Dublin causes diarrhea, dysentery, septicemia, fever, and abortion in cows and S. Choleraesuis causes septicemia, skin discoloration, and fever in pigs (Bäumler et al., 1998). S. Dublin and S. Choleraesuis are commonly associated with bacteremia opposed to diarrhea in humans (Blaser et al., 2001). S. Typhimurium causes a typhoid-like systemic illness in mice, but S. Typhimurium infection in humans is typically limited to gastroenteritis (Bäumler et al., 1998). The mechanisms of adaptations and disease variability among host species is a complex phenomenon that is dependent on a large number of gene products that are incompletely understood (Evangelopoulou et al., 2013).

S. Typhi and S. Paratyphi are the two serovars that cause classic enteric fever in humans. This is a systemic illness with early clinical manifestations of fever, abdominal pain, transient diarrhea or constipation, and occasionally a maculopapular rash. The hallmark sign of enteric fever is mononuclear cell infiltration and hypertrophy of the reticuloendothelial system affecting intestinal Peyer’s patches, mesenteric lymph nodes, the spleen, and bone marrow (Ohl and Miller, 2001). In untreated patients, fever persists for two weeks or more with recovery requiring 3-4 months. Among untreated patients, 10%-15% experience including intestinal hemorrhage (due to erosion of necrotic Peyer’s patches); intestinal perforation; and typhoid encephalopathy (Parry, 2006).

Non-typhoidal Salmonella serotypes cause gastroenteritis that remains localized to the terminal ileum, colon, and associated mesenteric lymph nodes. Following oral ingestion, the incubation period averages < 1 day (Sterzenbach et al., 2013). The salmonellae invade the terminal ileum and colon, resulting in an inflammatory tissue response. Necrosis and mucosal sloughing follow, along with neutrophils present in the feces (Harris et al., 1972). The host’s inflammatory response is responsible for the symptoms of diarrhea, nausea, vomiting, intestinal cramping, and fever.

The gastroenteritis caused by Salmonella serovars in other large animal mammalian species is similar to non-typhoidal disease in humans. Animals are typically exposed through the fecal-oral route either directly or by contaminated food and/or water. Recirculation of these pathogens in the environment allows them to persist in their respective animal reservoirs. Many Salmonella serovars can cause gastroenteritis, but only a select few serovars account for the
majority of cases in any given animal reservoir (Sterzenbach et al., 2013). It is from these animal reservoirs and replicative hosts that serovars move to additional susceptible host species.

1.4. *Salmonella* Virulence Factors Associated with Gastroenteritis

1.4.1. Surface Structure – The “O” Antigen

Gram-negative bacteria contain a cell envelope that consists of a peptidoglycan layer, capsule, flagella, pili, inner membrane (IM), and an outer membrane (OM). Between the inner and outer membranes is an aqueous cellular compartment called the periplasm. The periplasm is densely packed with a variety of soluble proteins including degradative enzymes, redox carriers, and chaperone-like molecules that are important for envelope biogenesis (Silhavy et al., 2010). The IM is a phospholipid bilayer that contains many of the membrane proteins that function in energy production, lipid biosynthesis, protein secretion, and transport (Silhavy et al., 2010). The peptidoglycan layer is an essential structural component that helps preserves cell integrity by withstanding osmotic pressure, gives the cell a defined shape, and is the scaffold for anchoring other cell envelope components such as proteins (Vollmer et al., 2008).

The outer most part of the Gram-negative cell is the OM. The critical function of the OM is to serve as a selectively-permeable barrier. The OM is an asymmetrical bilayer that contains phospholipids and lipopolysaccharides (LPS). The OM is built as a bilayer of lipids that contain mainly channel-forming membrane proteins, called “porins”, that allow influx of nutrients and the excretion of waste products (Nikaido, 2003).

The LPS consists of three structural regions: a hydrophobic region called lipid A (or endotoxin), the nonrepeating core oligosaccharide, and the distal O polysaccharide (or the O-antigen). LPS is anchored into the OM by the hydrophobic lipid A. The structure of core oligosaccharide is highly conserved among *Enterobacteriaceae* and serves as an attachment site for the enterobacterial common antigen (ECA) or the variable O-antigen (Raetz and Whitfield, 2002). The ECA is not fully understood, but may have a role in bile salt resistance (Bridge et al., 2015). LPS is also important in bile salt resistance and may provide an effective barrier to limit access of bile salts to the OM (Crawford et al., 2012; Merritt and Donaldson, 2009; Prouty et al., 2002; Spector and Kenyon, 2012). The O polysaccharide at the cell surface is placed at the interface between the bacterium and its environment. Due to this exposure to the environment and its inherent antigenicity, the O polysaccharide defines O-antigen serological specificity. There are many unique O-antigens for *Salmonella enterica* with 46 serogroups and many
additional modifications to the O-repeat unit which create additional “O factors” (Raetz and Whitfield, 2002).

The O-antigen is the major unit of LPS used in the WKLM naming scheme. Each serogroup is identified by a major “O-antigen(s)” found only in that particular serogroup. These Salmonella serogroups are designated by letters. An example is the “4” O-antigen, which is the representative antigen for serogroup B and cannot be found in any other serogroup. Group D has the “9” O-antigen as the representative antigen, but is further divided into serogroup D₁ with the “9” O-antigen, serogroup D₂ with antigens “9,46”, and serogroup D₃ with antigens “9,46,27” (Grimont and Weill, 2007). The variability and diversity of the O-antigen subunit lengths and sugar compositions from strain to strain form the basis for serotyping (Lam et al., 1989). The O-antigen chains confer complement-mediated serum resistance because the membrane attack complex forms at a great distance to the cell surface and fails to insert into the bacterial outer membrane (Bravo et al., 2008; Islam and Lam, 2014).

1.4.2. Surface Structure – The “H” Antigen

Flagella are the motility structures of Salmonella and the flagellin protein is known as the “H-antigen” (Wilson et al., 2011). Among Enterobacteriaceae, Salmonella is unique in that it commonly has two distinct H-antigens, phase 1 (H₁-antigen) and phase 2 (H₂-antigen) flagellar proteins (McQuiston et al., 2004). The bacterium has the ability to change its flagellar proteins to avoid being cleared by the host’s immune system (Wilson et al., 2011). The H-antigens of Salmonella have been well described and are primarily encoded by one of two genes, fliC and fljB, which express the phase 1 H₁-antigen and the phase 2 H₂-antigen, respectively (Wilson et al., 2011). The fliC gene is located in one of the flagellar biosynthesis operons and is present in all Salmonella. The fljB gene is located in a unique region of the genome in Salmonella enterica and is present in four of the S. enterica subspecies (subspecies I, II, IIIb, and VI) (McQuiston et al., 2011). Salmonella is able to switch between phase 1 and phase 2 H-antigens by the coordinately regulated expression of the two flagellin loci fliC and fljB so that only one phase is expressed at a time in a single cell (McQuiston et al., 2011; Wilson et al., 2011). Serovars expressing two flagellar antigen types are called diphasic, while those with only one flagellar antigen type are considered monophasic. Genes encoding flagellin are switched by DNA invertase, called HIN, which promotes inversion of the sequences and phenotypic switching (Wilson et al., 2011). Subspecies IIIa, IV, VII and S. bongori do not contain the fljB operon and
are historically monophasic (McQuiston et al., 2011). Variable regions of the fliC and fljB alleles have led to immunologically different H-antigens. Differences which correlate to these antigen protein sequences can differ in as few as one amino acid residue (McQuiston et al., 2004).

1.4.3. **Surface Structure – The Capsular or “Vi” Antigen**

A select few serovars are able to produce a capsular polysaccharide composed of N-acetylglucosamine uronic acid. This capsular antigen is called the Vi (‘virulence’) antigen and is produced by *Salmonella Typhi*, *Salmonella Dublin*, and *Salmonella Paratyphi C* (Gunn et al., 2014; Seth-Smith et al., 2012). The Vi-antigen capsule is thought to enhance systemic virulence by: increasing bacterial resistance to complement, reducing phagocytic killing by protecting the bacterium from reactive oxygen species (ROS), and interfering with pathogen-associated molecular patterns (PAMPs) activation of the innate immune system (Gunn et al., 2014). In all three serovars, the Vi-antigen is encoded on the viaB locus within the *Salmonella* Pathogenicity Island-7 (SPI-7) and is controlled by the RcsB–RcsC and OmpR–EnvZ two-component regulatory systems (Gunn et al., 2014; Pickard et al., 2003). SPI-7 is the largest genomic island identified in *Salmonella* and comprises regions thought to be involved in the island’s mobility, as well as regions responsible for the production and export of the Vi-antigen, SopE phage, and a type IVB pilus locus (Seth-Smith, 2008). When the viaB locus is activated, the flagellar master regulator flhDC is repressed by the TviA regulatory protein and flagella production is halted (Winter et al., 2008).

SPI-7 is a modular genetic island with an unusually high degree of horizontal genetic exchange and appears to have arisen through serial acquisition of functional units (Seth-Smith, 2008). A detailed sequence analysis of *Salmonella Typhi*, *Salmonella Dublin*, and *Salmonella Paratyphi C* by Pickard *et al* showed that the SPI-7 sequence is has at least a 99% similarity at the DNA and protein levels. The major difference is that the SopE phage is located on SPI-7 in *S. Typhi*, but is not found in SPI-7 in *S. Dublin* and *S. Paratyphi C*. It is thought that the SopE phage may have been acquired by serovar Typhi after the basic SPI-7 element was acquired because the SopE phage is still capable of excision from SPI-7. Other than this, only a small number of deletions or insertions have been found among the three SPI-7 elements. Pickard *et al*. (2003) believes that the original SPI-7 was most similar to that found in *S. Dublin* and that there was a common source of SPI-7 for all three serovars. They suggest that because DNA sequences of the common regions of SPI-7 are highly conserved and suggests that SPI-7 acquisition by the
three serovars was a relatively recent event. They also state that the analysis of the overall gene complement of SPI-7 provides additional evidence that SPI-7 was originally obtained by horizontal transfer, perhaps in the form of a conjugative transposon by the type IVB pilus which could have originally constituted the mating pair formation system for a conjugative transposon or plasmid (Pickard et al., 2003).

1.4.4. Pathogenicity Islands and Pathogenesis in Mammalian Epithelial Cells

After a *Salmonella* cell has been ingested, there are several virulence factors that allow it to (a) invade the host’s intestinal epithelium, (b) elicit inflammatory changes in the intestinal epithelium, and (c) evade neutrophil-mediated killing that is essential for pathogenesis (LaRock et al., 2015). *Salmonella* species (spp) have 24 SPI’s which encode the genes for these virulence factors (Hayward et al., 2013; Sabbagh et al., 2010; Urrutia et al., 2014). SPI-1 through SPI-5 are common among all *S. enterica* with SPI-1 and SPI-2 being the two conserved and stable pathogenicity islands (PAI’s) found in all *S. enterica* (Hurley et al., 2014; Nieto et al., 2016). The major virulence factors in *Salmonella* spp during the infection process are the injectosome, type III secretion system 1 (T3SS-1) and T3SS-2, encoded within SPI-1 and SPI-2, respectively (Ryu et al., 2014). The T3SS-1 is primarily associated with invasion, while the T3SS-2 primarily secretes effector proteins which promote the intracellular survival of *Salmonella* spp (Hallstrom and McCormick, 2011).

After surviving the acidity of the gastric portion of the GI tract, the bacterium enters the small intestine, where it binds to epithelial cells overlying intestinal lymphoid tissue (Holt, 2000). After binding, T3SS-1 mediates irreversible adhesion after which a series of secreted effector proteins are injected into the host cell cytosol (Keestra-Gounder et al., 2015; Que et al., 2013). These proteins mediate uptake of the salmonellae due to host membrane “ruffles” and cytoskeleton rearrangements (Que et al., 2013). After traversing the epithelial layer, the pathogen is phagocytosed by macrophages where it persists within a “*Salmonella* spp containing vacuole” (SCV) by preventing vacuole maturation and fusion with lysosomes (Nieto et al., 2016). SPI-1 genes mediate the SCV development (Que et al., 2013). The *Salmonella* spp can proliferate once the SCV appears in the perinuclear region.

SPI-2 is important for intracellular growth of *Salmonella* spp and for maintaining the integrity of the SCV (Figueira and Holden, 2012; Hurley et al., 2014). From within the SCV, SPI-2 genes encode the T3SS-2, which enables *Salmonella* spp to translocate a range of effector
proteins into the cytoplasm of the host cell (Hurley et al., 2014). Effector proteins from SPI-2 allow the SCV to capture nutrients from endocytic and exocytic transport vesicles. Once enough nutrients have been acquired, the bacterium starts to replicate. The host cell mounts a defense by acidification of the phagosome lumen, activation of cationic proteins, and production of antimicrobial peptides such as defensins. The pathogen responds by activation of a two-component regulatory system, PhoP/PhoQ, which is involved in acid tolerance and resistance to cationic peptides. The PhoP/PhoQ system controls expression of SPI-2 effector proteins, which in turn reduces the exposure of the salmonellae to the battery of host defenses, i.e., antimicrobial peptide defense, antigen presentation and reactive oxygen and nitrogen species generation (López et al., 2012). The SPI-1 T3SS effectors trigger production of proinflammatory cytokines and the less well-characterized SPI-2 T3SS-2 proinflammatory activity, which stimulates rapid recruitment of neutrophils and induces acute intestinal inflammation and gastroenteritis (Andrews-Polymenis et al., 2010; Figueira and Holden, 2012). This response is exacerbated by SPI-1-dependent induction of macrophage cell death (Figueira and Holden, 2012). The host cell death is induced by SPI-1 effectors as well as SPI-2 effectors; this results in programmed cell death and further dissemination of salmonellae (López et al., 2012).

1.4.5. *Salmonella and the Microbiota*

The gut is an anaerobic environment that requires microbes to rely on fermentation to produce energy for growth. In the intestinal tract, normal bacterial flora produces an abundance of hydrogen sulfide (H₂S). Hydrogen sulfide is toxic to the intestinal tissue, which prompts the intestinal epithelial cells to detoxify it to thiosulfate. Host enterocytes produce enzymes which oxidize H₂S to thiosulfate (S₂O₃⁻), thereby protecting themselves from the toxic effects of H₂S (Winter and Bäumler, 2011).

Microbes depend on the nutrients present in the mucous layer for growth. In order to outcompete the microbiota, *Salmonella* spp must utilize nutrients generated as a consequence of the host inflammatory response. *Salmonella* spp require both motility and chemotaxis towards mucous carbohydrates to increase its abundance in the intestinal lumen. The pathogen uses fimbrial adhesions that bind carbohydrate moieties present in the mucous layer to colonize this niche (Santos et al., 2009).

During gastroenteritis, neutrophils transmigrate into the intestinal lumen in large numbers, giving rise to an abundance of fecal leukocytes, which are characteristic of
inflammatory diarrhea. These neutrophils generate ROS that oxidize thiosulfate ($S_2O_3^{2-}$) into tetrathionate ($S_4O_6^{2-}$). The ttrBCA ttrRS gene cluster codes for tetrathionate reductases enable Salmonella spp to use tetrathionate as a terminal electron acceptor (Hensel et al., 1999). Through this mechanism, inflammation provides a respiratory electron acceptor that enables Salmonella spp to use respiration instead of fermentation to produce energy for growth (Winter et al., 2010). Salmonella spp are able to use tetrathionate as an electron receptor in anaerobic respiration and this gives them an advantage in the intestinal environment. Tetrathionate respiration enables Salmonella spp to utilize fermentation end products that cannot be consumed by the fermenting microbiota. The use of tetrathionate respiration for energy production presents S. Typhimurium with an important growth advantage over competing microbes that rely on fermentation. Inactivation of genes required for tetrathionate respiration removes the ability of S. Typhimurium to outgrow the microbiota during intestinal inflammation (Winter et al., 2010). This data indicates that tetrathionate respiration is one of the main mechanisms enhancing outgrowth of Salmonella spp in the inflamed gut.

The pathogenic strategy of Salmonella spp associated with gastroenteritis is to use virulence factors (T3SS-1, T3SS-2, and others) to elicit acute intestinal inflammation. This host response provides a new respiratory electron acceptor in the gut, enabling the pathogen to outgrow the microbiota in the lumen, thereby enhancing its transmission to the next host by fecal shedding of the organism. Salmonella spp thus use the host to provide them with a substance that allows them to outgrow their competition (Bäumler et al., 2011).

Salmonella spp have evolved ways to subvert, mimic, antagonize, and exploit the defense strategy of vertebrate hosts with their virulence factors creating a novel-niche that favors growth of Salmonella spp, in order to outcompete the resident microbiota (Hallstrom and McCormick, 2011; Rivera-Chávez and Bäumler, 2014). Salmonella spp residing in the tissue face death by the host’s innate immune system, but acute inflammation actually changes the environment of the gut lumen to favor Salmonella spp growth (Rivera-Chávez and Bäumler, 2014). Luminal outgrowth to increase their abundance in intestinal contents during gastroenteritis is required for successful transmission to the next naïve host by the fecal-oral route. Diarrheal disease
(gastroenteritis) flushes the intestinal lumen thereby removing the intestinal contents and the salmonellae (Rivera-Chávez and Bäumler, 2014; Sterzenbach et al., 2013).

1.5. Bacteriophage

1.5.1. Background

Bacteriophages are viruses which infect bacteria. Bacteriophages (phages) can be used therapeutically as highly specific antimicrobial agents; also called “phage therapy” (Monk et al., 2010).

In 1896, Ernest Hankin, a British bacteriologist, reported an unidentified substance (which passed through fine porcelain filters and was heat labile) in the waters of the Ganges and Jumna rivers in India which was responsible for antimicrobial activity that appeared to prevent the spread of the \( \text{Vibrio cholerae} \) bacterium and limited the spread of cholera epidemics (Borie et al., 2014; Sulakvelidze et al., 2001). A medically trained English bacteriologist named Frederick Twort published the first report of viruses that infect bacteria in 1915, noting that they replicated within and killed the bacterial cells (Rohwer and Segall, 2015; Twort, 1915).

In 1917, the “bacteriophage” era began with a seminal publication by the French-Canadian microbiologist Félix D’Hérelle which demonstrated an “anti-microbe” of \( \text{Shigella} \) (D’Hérelle, 1917). D’Hérelle devised the term “bacteriophage” which means bacteria-eater to describe this property (Salmond and Fineran, 2015). In 1919, D’Hérelle used phages to successfully treat dysentery in a 12-year-old boy as the first recorded attempt to use bacteriophages therapeutically (Sulakvelidze et al., 2001).

D’Hérelle also tested the therapeutic utility of phages on bacterial pathogens of a number of different animals. He challenged flocks of chickens with \( \text{Salmonella Gallinarum} \) and successfully treated them with oral administration of phage. He successfully treated rabbits challenged \( \text{Shigella dysenteriae} \) with phage as well as found that phage-treated water buffaloes were protected from subsequent experimental inoculation with \( \text{Pasteurella multocida} \) (D’Hérelle, 1926).

D’Hérelle worked to determine the safety of bacteriophages before treating humans. He decided the best way to perform a human safety trial was to administer Shiga-bacteriophage treatment both orally and subcutaneously to himself, his family members, and his coworkers. He found no adverse effects, and subsequently began administering Shiga-bacteriophage to patients with culture confirmed bacillary dysentery. D’Hérelle’s most notable report had to do with
treatment of four cases of bubonic plague with *Yersinia pestis* phage. D’Hérelle treated all four patients with *Yersinia pestis* phage preparations by direct injection of phage into the buboes. All four patients recovered remarkably fast and the results were reported in the French medical periodical, *La Presse médicale* (Summers, 2001).

The Council on Pharmacy and Chemistry, established in 1905 by the American Medical Association to set standards for drugs and lead the battle against nostrums, undertook the evaluation of phage therapy in the late 1930s. The voluminous report of the Council, authored by Stanhope Bayne-Jones, a microbiologist, and Monroe Eaton, an infectious disease specialist, concluded there was an ambiguous assessment of the literature on efficacy of phage therapy and expressed concerns regarding the lack of understanding of the true nature of bacteriophages (Housby and Mann, 2009; Summers, 2001). They were also concerned about a lack of standardization for phage preparations as well as a lack of criteria for purity and potency which made it impossible for most of the published studies to be directly compared (Summers, 2001). The key conclusions of the report were that bacteriophages were not viruses capable of parasitizing bacteria, D’Hérelle’s theory that the material is a living virus parasite of bacteria had not been proved, and that facts appeared to indicate that the material was inanimate, possibly an enzyme (Sulakvelidze and Kutter, 2004). It was not until 1939 that Emory L. Ellis and Max Delbrück demonstrated that phages propagate at the expense of their host by performing their classic one-step growth curve experiment. They showed that a single phage particle infected a bacterial cell, multiplied inside the host until the viral progeny lysed the cell and were released into the environment (Ellis and Delbrück, 1939). This was further substantiated in 1940 by Helmut Ruska who used the electron microscope to directly observe and demonstrate phage particles adhering to a bacterial membrane; this helped to convince skeptics that the effects attributed to phages were not enzymatic but were indeed caused by viruses (Pennazio, 2006; Rohwer and Segall, 2015).

### 1.5.2. Life Cycle of Lytic Phages

It is now well established that phages are obligate intracellular parasites of bacteria. Like many other viruses, their replicative life cycle takes place within host cells, using bacterial cellular metabolism for the production of new phage particles, release of those particles from their cellular confines, and then is repeated with the infection of new cells (Hyman and Abedon, 2010). Phage lytic infection is initiated by the phage adsorption or binding to a specific receptor
on the surface of the host bacterium. After the phage is attached to the host cell, the phage genome is released into the cytoplasm. The phage genome takes over the host and uses the host machinery to replicate the viral genome and synthesize proteins that will assemble to produce the mature phage progeny. Specific viral lysins facilitate the release of the virions by lysis of the host bacterial cell, resulting in cellular death (Domingo-Calap et al., 2016; Hyman and Abedon, 2010). This final step of the lytic life cycle, in which phages kill their host bacterial cells, provides the cornerstone concept for using phages as therapeutic agents (Skurnik and Strauch, 2006). This replication cycle is why the term “self-replicating pharmaceuticals” was coined for phage therapy (Abedon and Thomas-Abedon, 2010).

1.5.3. Advantages and Disadvantages of Phage Therapy

For decades, extensive studies of bacteriophages for treatment of both acute and chronic infections were performed in countries of the former Soviet Union, with Georgia as the epicenter of phage study research (Sarhan and Azzazy, 2015). The focus of this discussion is on recent phage therapy research which is accessible in English-language publications and primarily from Western countries. For therapeutic uses, bacteriophages that are lytic are highly desirable because of rapid killing of their targeted bacterial host cell, which leads to phage number increases and minimized chances of transduction, horizontal gene transfer within the bacterial population by host DNA packaged into phage particles instead of the phage genome (Monk et al., 2010). Phages that belong to the viral order *Caudovirales* are the choice for phage therapy. *Caudovirales* is divided morphologically into three families, based on their tail length and complexity, called *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Kropinski, 2006; Monk et al., 2010). Phage virions may target their bacterial host by recognizing a number of cell-surface components, such as pilin or flagellin proteins, as well as other proteins, capsules, lipopolysaccharides, and teichoic acids (Lindberg, 1973). Determining the specific phage receptor and the phage host range is important when selecting a phage for phage therapy. For example, the lambdoid coli phages (*Siphoviridae*) may be unsuitable due to phage lambda being the prototype of temperate phages that can establish a lysogenic state. The T4 and T4-like phages (*Myoviridae*) are better choices because they destroy bacterial DNA early in the infection cycle. Nucleolytic attack seals the host cell’s fate and releases the nucleotides that are necessary for phage DNA synthesis within the infected cell (Brüssow, 2010).
Phage therapy is described in terms of the number of phage types used during treatment. Monophage therapy involves the use of only a single phage whereas polyphage therapy uses more than one phage simultaneously to create a phage “cocktail”. Phage cocktails broaden the utility of phage formulations by treating a selection of bacterial diseases and as a way of preventing the development of phage-resistant mutants during individual treatments (Chan et al., 2013).

A potential downside of phage therapy is that by using phages therapeutically, there may be pressure on the system that selects for bacteria that do not have the receptor(s) which the phage binds to, thus selecting for phage-resistant mutants of the bacteria. One negative outcome results when a bacterium undergoes lysogenic conversion following infection with a temperate phage, which confers immunity to that bacterial cell to the phage and its relatives, resulting in phage resistance. Another major negative side effect could be the transduction of additional virulence or antibiotic resistance genes from this lysogeny. However, it has been hypothesized that not all development of phage-resistance is necessarily a bad thing because such mutations may also lead to reduced fitness of the bacteria. For instance, if the receptor used by the phage is a virulence determinant, then receptor loss would consequently decrease the bacterium virulence (Skurnik and Strauch, 2006).

1.5.4. Mechanisms of Delivery and Pharmokinetics

There are different delivery mechanisms of bacteriophages. Parenteral (e.g., intravenous, inhalation, intraperitoneal, subcutaneous, or intramuscular) delivery in experimental animal studies is one of the most popular and successful delivery methods because of the rapid distribution of phages into the systemic circulation. The specific route of administration can heavily influence the success of the phage therapy. Other routes of delivery include oral delivery, topical administration, otic phage administration, dental phage administration, inhalation of phage, and phage pre-treatment of medical devices to prevent biofilm formation. The choice of delivery mechanism is dependent on the source of infection and all methods have been shown successful in treating pathogens ranging from *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumonia*, and *Salmonella* species to *Escherichia coli* (Ryan et al., 2011). For systemic infections, the most successful routes for phage therapy administration are the parenteral ones, with intraperitoneal administration of phages as particularly effective. Oral delivery has proven very successful with bacterial gastrointestinal infections and inhalation
delivery for lung infections. Studies on non-aqueous formulations represent a primary gap and priority area of need in phage therapy research (Ryan et al., 2011). Phages can penetrate circulate with the blood and gain access to organs and tissues very freely following all routes of parenteral administration. It bears repeating that at sites of the targeted bacterial infection(s), phages will continue to multiply and retain full biological activity so long as their host bacteria are present (Dabrowska et al., 2005).

The pharmaceutical potential of phages is a matter of some complexity: first, because phages are huge compared to antibiotic molecules, and thus can elicit an immune response; secondly, there must be a sufficient number of phages administered to the patient in order to reach the locations of bacterial infections; and thirdly, because phages vary in their virulence to their bacterial host. The presence of phages can non-specifically stimulate the immune system, thereby helping to combat the bacterial infection, or may initiate an immune response to the phage itself, which would lead to the phage being cleared by both the humoral and innate immune systems and thereby nullifying its intended therapeutic action (Dabrowska et al., 2005; Nilsson, 2014). So far, no immunological complications (anaphylaxis) have been documented following treatment with purified phage. Phage therapy may be density dependent because, without enough phages to reach the targeted bacteria, they may just be cleared from the system. Adsorption rates, latency times, and burst size all can affect the phages bacterial virulence, and therefore its therapeutic efficacy. All of these factors affect the pharmacokinetics of phage therapy (Nilsson, 2014).

The pharmacokinetics, toxicology, and clearance of phage from phage therapy have been reported. Bogovazova et al (1991, 1992) evaluated the safety and efficacy of phages for treatment of *Klebsiella* infections in mice and guinea pigs, using intramuscular, intraperitoneal, and intravenous administration of phages. Once phage is administered, whether intravenous, intramuscular, intraperitoneal, and even orally, they can be found throughout the mammalian circulatory system. Phages soon dissipate from the blood and organs unless a host bacterium is found. They persist for longer periods in the spleen compared to the blood stream or other organs. No signs of acute toxicity or gross or histological changes were observed in these animal studies even when a single dose was administered 3500-fold higher than the projected human dose. Following these animal safety trials, they successfully treated 109 patients with *Klebsiella* infections with no observed toxicity, marked clinical improvement (Bogovazova et al., 1991,
1992). Inchley (1969) using Chromium 51-labeled T4 bacteriophage injected intravenously found that more than 99% of the phages were phagocytized in the liver within 30 minutes after injection without a host bacterium.

Other studies with a host bacterium found that therapeutic phages could be found in the bloodstream for several days after oral administration of therapeutic phage (Babalova et al., 1968; Weber-Dabrowska et al., 1987); whereas in animals experimentally infected with phage and host bacteria, phages were found to enter the bloodstream 2 to 4 hours after intraperitoneal injection and was recoverable from internal organs (liver, spleen kidney, etc.) at 10 hours post injection (Bogovazova et al., 1991, 1992). Dubos et al (1943) on phage treatment in Shigella dysenteriae-infected mice, established (a) that the lytic effect of phages, and not an immune stimulant present in phage lysate, was responsible for the observed therapeutic effect, (b) phage can cross the blood-brain barrier by an unknown mechanism, and (c) that phages can locate and multiply in foci of bacterial infections anywhere in the body and phage can be found in circulation as long as there is a site of infection somewhere in the body (Dubos et al., 1943).

With the ever increasing concerns about development of antibiotic resistance among bacterial pathogens, phage therapy appears to have undeniable efficacy in the reduction of antibiotic resistant pathogens as well as significant potential for therapeutic uses. Phages have been shown to be effective in combating infections with little negative effects other than the possibly of the specificity of their host range. During the 20th century, medical and scientific papers from Soviet Union countries were not translated into English, hence the reason there has been a lack of knowledge about phage therapy in the Western scientific and medical community. Now a number of U.S. universities have microbiologists actively investigating phage therapy as a viable alternative to antibiotics, as well as for non-chemical sanitizing rinse applications in the commercial food supply. With time hopefully this will result in more effective and sustainable phage applications.

1.6. Bacteriophages and Host Interactions

Bacteriophage are viruses and like all other viruses, they are obligate intracellular parasites of cellular organisms. The basic life cycle of a lytic phage involves using cellular metabolism to produce new virus particles, release those particles, and then infect new cells. The viral life cycle must complete all three steps to be successful. The phage’s ability to successfully
complete the viral life cycle determines the host range of the bacteriophage (Hyman and Abedon, 2010).

The apparent host range of a phage is dependent on what technique and conditions are used to determine the host range. Plaquing, spot testing, or broth-based measurements of phage population growth are a few methods and can give varying results. These techniques employ different environments for the phage to be productive infecting a host. The first part of the host range is the receptor the phage is able to bind. Gram negative bacteria like *Salmonella* can have many different surface molecules that a phage may use as a receptor. Included are structures in the cell envelope including cell wall proteins, sugar moieties like components of the LPS, or the requirement of both structures for adsorption. Other cell envelope receptors include flagella, pili, and capsules (Hyman and Abedon, 2010). Recognition of the cell receptor is the first step in the infection process.

When the phage receptor in flagella-based, reversible binding occurs between the phage tail fibers and the flagella, or between flagella and the region connecting the head and tail of the phage, the phage is able to tumble down the flagella and move closer to the cell. Phages that use the connector region for adsorption use their tail fibers to find the receptor on the surface of the bacterium. Irreversible binding occurs when the virion bound to flagella use the tail fibers to adsorb to the baseplate of the flagella on the bacterial cell or finds the receptor on a neighboring cell (Lindberg, 1973; Rakhuba et al., 2010).

Bacteriophages also can use polysaccharides, LPS, and capsular antigens as receptors. Tail spike proteins can recognize and may be able to hydrolyze the LPS, allowing the phage to move through the LPS to the cell surface. Phages which recognize the Vi capsular antigen do so via a phage tail protein (Chaturongakul and Ounjai, 2014).

Hyman and Abedon (2010) have proposed seven categories for host-range interactions: adsorptive, penetrative, bactericidal, productive, plaquing, spotting, and lysogenic. In the adsorptive interaction, the phage is able to irreversibly adsorb to a host. Phage that use the penetrative interaction adsorb to the bacteria, as well as deliver DNA or RNA into the cytoplasm of the bacteria. The bactericidal interaction results in bacterial death and this can be observed with spot tests and plaque formation. In productive infections, the phage is able to produce and release phage from the host cell. Plaquing phages are a subset of productive phages which are able to form plaques on host cell lawns; not all hosts will allow phage plaquing. The spotting
interaction is a broader type bactericidal host range interaction, due to abortive infection mechanisms or lysis from without. Finally, lysogenic phages are able to form lysogens in their bacterial host (Hyman and Abedon, 2010).

1.7. Bacteriophage-Resistance of Microorganisms

1.7.1. Extra-cellular Mechanisms of Resistance

Each category of host-range determination gives insight into areas in which a bacterium can evolve to become resistant to that phage. There may be a few different extracellular mechanisms of resistance the bacterium may develop at the adsorption step. Prevention of phage adsorption can be by blocking the phage receptor (Labrie et al., 2010). Bacteria can hide phage receptors behind barriers consisting of extracellular polymers. These barriers can sometimes block phage infections, but may not always succeed in doing so (Hyman and Abedon, 2010). Phase variation and altering the surface is another method of hiding the phage receptor. 

*Salmonella* has two phases of flagella production. The phage may recognize the phase 1 H1-antigen flagellar antigen and not the phase 2 H2-antigen flagellar antigen, or vice versa (Shin et al., 2012).

The bacteria-encoded cell surface phage receptor molecule may be lost. The receptors may serve essential roles that can be costly for the bacteria to downregulate or delete (Hyman and Abedon, 2010). For instance, if the phage is adsorbing to a pathogenicity factor, this receptor loss will simultaneously attenuate the bacterium (Filippov et al., 2011).

Loss of the receptor may not be a requirement for adsorption phage resistance. An alteration in the receptor’s structure may affect the phage binding. A study of *Yersinia pseudotuberculosis* phage mutants showed duplications in the sequence for T4 phage tail fiber resulting in longer tail fibers. T4 typically infects *Escherichia coli* (*E. coli*), but does not infect *Y. pseudotuberculosis* efficiently. The duplication mutants adsorbed rapidly to the *Yersinia*, possibly due to the fact that the longer tail fibers were able to reach the receptor, suggesting that fiber length can limit the host range (Tétart et al., 1996). If the LPS or some other extracellular structure is lengthened, this may prevent the phage tail fibers from reaching its receptor. A modified LPS can also affect the phage binding. If the O-antigen structure that a phage recognizes is changed, then the phage cannot adsorb (Steinbacher et al., 1997). Modifications of surface receptors is the most frequent event causing phage resistance due to the potential that
only a single point mutation in a surface structure gene can cause a change in that structure and is the most probable source of resistance during phage therapy (Nilsson, 2014).

1.7.2. Intra-cellular Mechanisms of Resistance

Not all mechanisms of phage resistance are extracellular, but some can be intracellular. Phage resistance is a mechanism that prevents the completion of the viral life cycle in the bacterial host. A phage may be able to adsorb to a bacterium, but unless it is able to produce and release viral particles, the viral life cycle is not complete. These mechanisms of intracellular phage resistance can occur at the viral DNA or RNA uptake step, or by mechanisms that destroy viral DNA or RNA, or result in abortive infections, or prevent release of phage progeny (Hyman and Abedon, 2010).

Preventing the phage nucleic acid from overriding the host transcription/translation machinery and not allowing the replication of phage is a form of phage resistance (Hyman and Abedon, 2010). Phage T4 normally adsorbs to *E. coli* and degrades the peptidoglycan layer and an inner-membrane protein transduces the DNA into the cytoplasm. Phage T4 encoded protein Imm prevents the phage DNA from other T-even-like phages from translocating to the cytoplasm. Genes for proteins similar to this have been found in prophages, which are lysogenic. Expression of Imm-like proteins by prophages would allow a bacterium to be resistant to a similar phage (Labrie et al., 2010).

Restriction-modification systems are found in over 90% of bacterial genomes. Methylation of host DNA by methyltransferase protects it from restriction endonuclease cleavage. Phage DNA that enters the cytoplasm that is not methylated will be cleaved at specific recognition sites that the restriction enzyme recognizes. The more restriction sites a phage genome has, the more susceptible the phage DNA is to the restriction enzyme (Dupuis et al., 2013).

The CRISPR-Cas systems found in bacteria are a form of immune system for the organism. A bacterium that has survived infection, will have small sections of invading DNA added to the CRISPR locus. The CRISPR transcript is produced and Cas proteins cleave repeats to produce smaller RNA or crRNA. The mature crRNA and Cas proteins recognize sequence-specific invading DNA and degrade it to protect the cell (Dupuis et al., 2013).

If the phage DNA makes it into the cytoplasm safely and is able to take over the host cell, the phage resistant mechanism of abortive infection (Abi) systems can protect the rest of the
population of the bacterium (Hyman and Abedon, 2010). Abi systems function as toxin-antitoxin systems which contain a toxin and an antagonistic antitoxin. These toxin-antitoxin systems can be interactions between RNA-RNA, protein-protein, or protein-RNA. Antitoxins are labile compared to their toxins. When production of both components are inhibited, the antitoxin concentration decreases more rapidly than the toxin concentration. Known Abi toxins can target cellular processes like DNA replication and translation by inhibiting DNA gyrase and causing mRNA degradation (Fineran et al., 2009).

The Rex system in *E. coli* phage λ is a two-component regulatory abortive infection system. After phage DNA enters the cytoplasm, a protein-DNA complex forms that activates RexA. Two activated RexA proteins mobilize the membrane protein RexB. RexB is a membrane ion channel that reduces membrane potential (Labrie et al., 2010; McGrath et al., 2002). Altruistic cell death occurs by hydrolysis of cellular ATP, thereby decreasing the synthesis of macromolecules and stopping cell multiplication, which results in cell death (McGrath et al., 2002; Parma et al., 1992). These Abi systems allow the phage-infected cells to altruistically commit suicide to protect the clonal bacterial populations (Blower et al., 2011).

### 1.7.3. Effects of Phage-Resistance on the Host Cell

Genes can be exchanged between bacteria by conjugation, transformation or transduction. Phage that can mediate transduction are readily isolated from the environment and are present in the animal intestine. Phage that enter a lysogenic state (called lysogenic conversion) contribute to a significant proportion of their host’s genome. These phages that are in the lysogenic state are called prophages (Thomson et al., 2004). Prophages can have an effect on the inhibition of other phages including themselves, and this is called “super-infection exclusion”. A protein named SieA is found in the inner membrane of *S. Typhimurium* infected with the lysogenic phage P22. The SieA protein prevents superinfection by *Salmonella* phages L, MG178, and MG40 (Hofer et al., 1995). In *P. aeruginosa*, prophage FIZ15 alters the O-antigen of its host and confers resistance to phage D3. *P. aeruginosa* lysogenized with prophage FIZ15 also has increased virulence by being more resistant to phagocytosis and increased adherence to epithelial cells (Vaca-Pacheco et al., 1999).

The *Salmonella rfb* locus encodes enzymes responsible for the synthesis of the O-antigen polysaccharide (Whitfield, 1995). Other chromosomally encoded genes, which are not located in the O-antigen gene cluster, are often involved in the modification of the structure and
particularly in the addition of side-chain residues to the O units. Some prophages can encode genes that affect the side-chain residues. If the phage is mobile, (meaning it can be excised, go through a lytic life cycle, infect new bacterial hosts and integrate into the new host genome), then the specific side-branch modifications can be acquired by horizontal transfer. *Salmonella* serogroup E was subdivided into E₁, E₂, E₃, and E₄, but the groups E₁, E₂, and E₃ were regrouped as E₁ due to the variations in side-chains of the serogroup E O-antigen as the result of side-chain modification genes on different bacteriophages. This is similar to *Salmonella* serogroups C₂ and C₃ that have been reclassified as serogroup C₂-C₃ due to the differences being a side-chain modification from a bacteriophage-encoded set of genes (Liu et al., 2014). Modifications to the LPS of *Salmonella* spp by lysogenic conversion could alter the LPS enough that certain phage could not infect the *Salmonella* spp if the modified LPS was the phage’s receptor site (Rapin and Kalckar, 1971).

1.8. **Salmonella Epidemiology in Humans**

*Salmonella* spp infections affect roughly a million people in the USA each year. The CDC estimates that *Salmonella* spp infections can range from 645,000 to 1.7 million cases a year, but only approximately 42,000 cases are laboratory confirmed and reported to the surveillance system (Scallan et al., 2011). CDC Foodnet Annual Report for 2015 has *Salmonella* spp ranked highest among laboratory confirmed bacterial foodborne pathogens with an attack rate of 15.89 per 100,000 population (e.g., 15.89 x 10⁶) (Huang et al., 2016). The United States Department of Agriculture (USDA) Economic Research Service reports that in 2013, *Salmonella* spp were responsible for approximately 11% of all foodborne illnesses, second only to Norovirus infections. Non-typhoidal *Salmonella* spp is estimated as the leading cause of hospitalizations (35% or 19,000 cases) and deaths (28% or 378 deaths) caused by foodborne illness linked to a specific pathogen. *Salmonella* spp are ranked first among 15 pathogens in terms of economic burden, estimated at $3.7 billion in a typical year. Ninety percent of the burden to deaths ($3.3 billion), 8 percent due to hospitalization ($294 million), and the remaining 2 percent to non-hospitalized cases. The economic burden can range from $193 million to $9.5 billion annually (Hoffmann et al., 2015).

In 2013, the CDC reported there were 45,735 laboratory-confirmed human *Salmonella* spp infections. Approximately 25% of these labator-confirmed *Salmonella* spp infections were in children 4 years of age or younger. The most frequently reported serovars were *S. Enteritidis*
(15.1%), S. Typhimurium (12.8%), S. Newport (8.3%), S. enterica serovar I 4,[5],12:i:- (5.3%), S. Javiana (5.0), S. Heidelberg (3.1%), S. Infantis (2.3%), S. Saintpaul (2.3%), S. Muenchen (2.1%), and S. Montevideo (2.0%). Most of these serovars have fluctuated in prevalence since 2003 with the exceptions of S. Typhimurium (decreasing, -13.5%) and S. enterica serovar I 4,[5],12:i: (increasing, +337.3%). S. enterica serovar I 4,[5],12:i:- had the largest increase at 337% since 2003, but the increase is partially due to increased laboratory recognition and changes in surveillance (reporting practices) (CDC, 2016). The decline in incidence of S. Typhimurium may be attributable to the use of a live attenuated S. Typhimurium vaccine in poultry and more stringent performance standards for prevention of Salmonella spp contamination of poultry carcasses (Huang et al., 2016). The US Department of Health and Human Services’ “Healthy People 2020” made it a national health objective to reduce Salmonella spp infections by 25% by the year 2020. This was because Salmonella spp infections were causing the largest number of illnesses, hospitalizations, and deaths of any bacterial pathogen in the Foodborne Diseases Active Surveillance Network (FoodNet) (CDC, 2011). The 2020 objective target is an incidence rate of 11.4 per 10^6 people; in 2015 the incidence rate was reported as 15.89 per 10^6, which is unchanged from the 2006-2008 report (CDC, 2011; Huang et al., 2016).

1.9. Salmonella in Animals

Salmonella spp is not just a human pathogen, but is capable of causing disease in other species, especially domestic livestock and poultry. In 2012, the top ten laboratory-confirmed serovars isolated from clinical non-human sources submitted to the National Veterinary Services Laboratories (NVSL) were Typhimurium (19.8%), Dublin (5.3%), Agona (5.1%), Derby (4.8%), Newport (4.8%), Cerro (3.9%), Enteritidis (3.8%), Infantis (3.5%), S. enterica serovar I 4,[5],12:i:- (3.4%), and Heidelberg (3.2%). The top ten laboratory-confirmed serovars isolated from non-clinical non-human sources submitted to the NVSL were Kentucky (14.9%), Enteritidis (13.2%), Heidelberg (11.9%), Senftenberg (9.1%), Typhimurium (6.0%), Mbandaka (4.5%), Montevideo (2.1%), Muenster (2.1%), and Braenderup (2.0%). The top 5 laboratory-confirmed serovars isolated from bovine clinical sources submitted to the NVSL were Dublin (18.8%), Cerro (13.5%), Montevideo (8.2%), Typhimurium (7.4%), and Newport (6.1%). The top 5 laboratory-confirmed serovars isolated from clinical equine sources submitted to the NVSL were Typhimurium (15.7%), Newport (15.5%), Typhimurium var. 5- (15%), Anatum (5%), and
Norwich (4.8%). The top 5 laboratory-confirmed serovars isolated from non-clinical bovine sources submitted to the NVSL were Montevideo (16.3%), Kentucky 13.9%), Typhimurium var. 5- (12.7%), Cerro (10.4%), and Anatum (6.8%). The top 5 laboratory-confirmed serovars isolated from chicken clinical sources submitted to the NVSL were Enteritidis (57.9%), Kentucky (8.2%), Typhimurium (4.3%), Muenchen (3.4%), and Newport and Thompson (2.6%). The top 5 laboratory-confirmed serovars isolated from non-clinical chicken sources submitted to the NVSL were Heidelberg (16.6%), Kentucky (15.9%), Enteritidis (13.6%), Seftenberg (11.6%), and Mbandaka (6.9%) (CDC, 2014).

1.10. Environmental *Salmonella* spp

The incidence of *Salmonella* spp has been extensively studied in animals and environmental sites. There is a wide array of environmental niches in which *Salmonella* spp can survive. *Salmonella* spp may be disseminated in various water sources such as effluent discharges, agricultural runoff, excretions by wild animals, and fresh water. Sediments may protect enteric organisms like *Salmonella* spp from stresses in aquatic environments and provide some nutrients. Water contaminated with animal waste has the potential for proliferation and dissemination of *Salmonella* by wild animals (Murray, 2000).

Farm environments can easily be if there are outbreaks of *Salmonella* spp among animals or some of the animals on the farm are asymptomatic carriers. Other than animal-to-animal transmission, additional factors for on-farm environmental spread of *Salmonella* spp includes recycling of (manure) lagoon waste water for flushing, contaminated feeds, inadequately controlled rodent and wild bird populations, contaminated rendering trucks being driven into animal areas and use of the same loader for transporting dead animals and moving feeds without appropriate cleaning and decontamination (Murray, 2000). Movement of animals can also lead to the spread of *Salmonella* spp by an introduction of an infected or carrier animals into a herd of non-infected animals (Wray et al., 1990). Since the sources for contamination of environmental sites are so diverse that absolute elimination of *Salmonella* spp in the outdoor farm environment is not possible, but addressing efforts to prevent introduction, minimize pathogen load, and prevent unintended distribution spread may assist in reductions into the food-chain (Murray, 2000).
1.11. Role of Carrier Animals

The host-host transmission of *Salmonella* spp is primarily by the fecal-oral route. After a host becomes infected, most of the time the host will resolve the salmonellosis and shedding will stop, but a few infected individuals will become carriers and will intermittently shed *Salmonella* spp in the feces for long periods of time. These animals can act as reservoirs for the pathogen and with meat-producing animals, these individuals can be sources of food contamination for humans by fecal contamination of vegetables, fruit, and nuts, or from fecal contamination of carcasses upon slaughter (Gopinath et al., 2012).

1.12. *Salmonella* in Cattle

Infections with *Salmonella* spp in cattle can cause mortality and morbidity. Subclinically infected cattle pose a threat for humans primarily by being a reservoir for the pathogen. Feces serve as the primary source from which *Salmonella* are transmitted via multiple routes. Transport vehicles may function as a source of exposure for cattle. Young calves can easily be infected during transport and may disseminate infection among calves or cattle at rearing farms or markets (Wray et al., 1991). Purchase of subclinically infected calves can lead to introduction of *Salmonella* spp into new herds (Wray et al., 1990). Alternative routes of infection can be aerosols from high pressure washers used for cleaning. *S.* Typhimurium can survive in the air for sufficiently long periods to present a potential hazard of airborne spread and can infect nearby calves (Wathes et al., 1988). Additional factors labeled as stressors may also exacerbate the disease or increase susceptibility. From the environment to fellow herd mates, cattle can be exposed to *Salmonella* spp from a wide range of sources.

With cattle, super-shedders shed $\geq 10^4$ CFU *Salmonella* per gram of feces and pose a risk of contamination in the food chain. These super-shedders can also be major contributors to environmental spread of zoonotic pathogens like *Salmonella* (Stanford et al., 2011). Calves exposed to cows with natural infections of *S.* Dublin, and calves housed in *S.* Dublin-contaminated buildings were found to be infected with *S.* Dublin. So exposure to environmental contamination of *Salmonella* spp can lead to infection (Wray and Sojka, 1981). An experiment to evaluate transmission of *S.* Typhimurium in a herd found that feeder calves were able to transmit *S.* Typhimurium to 80% of naïve calves within one week and 23% of those became asymptomatic carriers (Clinton and Weaver, 1981). Thus, it is critical to identify carriers and
properly manage sources of environmental contamination to prevent the spread of *Salmonella* spp in the farm environment.

*Salmonella* spp are a concern in the beef and dairy industries from both animal and human health viewpoints. A USDA *Salmonella* spp surveillance study in 1996 found that 20% (18/90) of dairy operations had at least one cow positive for *Salmonella* spp by fecal culture and 5.4% (194/3585) of cows sampled were *Salmonella*-positive. Culture-based herd prevalence increased from 1996 to 2007; in 2007 where 39.7% (48/121) of dairy operations had at least one cow positive for *Salmonella* spp by fecal culture and 13.8% (523/3804) of cows sampled were *Salmonella*-positive. Percentage of operations fecal-culture positive for *Salmonella* increased with the size of the dairy herd. In 2007, dairy herds with fewer than 100 cows were 24.3% *Salmonella*-positive, herds with 100-499 cows were 44.7% *Salmonella*-positive, and herds with 500 or more cows were 48.7% *Salmonella*-positive. From dairies sampled in 2007, *S*. Cerro, *S*. Kentucky, *S*. Muenster, *S*. Meleagridis, and *S*. Montevideo were the top 5 most prevalent serovars isolated from healthy cows (USDA, 2011). Cummings *et al*., found that 22.5% of samples from 93 herds were positive for *Salmonella* spp and 43% of herds had at least one case of laboratory confirmed salmonellosis (Cummings *et al*., 2009b). Another study by Cummings *et al*., found that cows may shed *Salmonella* spp for more than 1 year and fecal shedding frequently persisted after clinical signs of salmonellosis. They found that the serovar being shed was no predictor for duration of shedding (Cummings *et al*., 2009a). A study by Aboud *et al*., looked at prevalence of fecal shedding in cull cows from California dairies. They found prevalence of fecal shedding of *Salmonella* spp in cull cows was 3.42% (Aboud *et al*., 2016). A similar study by Wells *et al*., found total prevalence of *Salmonella* spp fecal shedding was 10% with dairy cows, 5.4% in milking cows, 18.1% in cows to be culled across USA dairies and 14.9% for cull dairy cows at markets across the USA (Wells *et al*., 2001). Studies by USDA of cattle feedlots in the USA found a *Salmonella* spp positive prevalence rate of 9.1% in fecal samples, 35.6% of pens, and 60.3% of feedlots (USDA, 2014).

1.13. *Salmonella* in Pigs

The top 5 laboratory-confirmed serovars isolated from clinical porcine sources submitted to the NVSL were Typhimurium var. 5- (20.7%), Derby (11.9%), Agona (9.9%), Infantis (6.0%), and Typhimurium (5.4%). For 2012, there were only 19 laboratory-confirmed serovars isolated from non-clinical porcine sources submitted to the NVSL with Typhimurium var. 5- accounting
for 36.8% of serotyped isolates (Centers for Disease Control and Prevention (CDC), 2014). In 2006, the USDA’s National Animal Health Monitoring System sampled swine production sites with 100 or more pigs. From 135 sites, sites with at least one positive Salmonella fecal sample were 52.6% of sites, 43.5% barns, and 18.4% pens. Of all the fecal samples collected, 7.2% of total samples were positive for Salmonella spp. Four main serovars accounted for 70.5% of isolates and were S. Derby, S. Typhimurium Copenhagen, S. Agona, and S. Anatum. The prevalence of Salmonella spp in swine feces in the U.S.A. is low, but more than half of sites have one or more positive fecal cultures (USDA, 2009).

The main mode of transmission of Salmonella spp in pigs is by the fecal-oral route, but it also may be transmitted through the respiratory tract. A study of alternate routes of invasion by Salmonella found that the tonsils and lungs are important sites for invasion by S. Typhimurium and that infection of Peyer’s patches may be due as much to blood-borne infection as to invasion from the gut (Fedorka-Cray et al., 1995). Wild animals may also be a source of spread in pig farms. An investigation of 23 pig farms found Salmonella spp in a variety of cats and wild animals such as rats, mice, wild birds, and foxes. Cats and birds were associated with contamination of feed and grain stores whereas rodents were involved in perpetuation of infection in specific buildings on the farms (Davies and Wray, 1997). Swine farms should utilize an all-in, all-out policy with adequate cleaning and disinfection after the pen is empty.

Biosecurity control measures on swine farms are critical to prevent introduction of Salmonella spp in swine facilities and to control spread of Salmonella spp within a facility. Biosecurity measures such as changes of clothing and boots for visitors, bird and rodent control, foot-baths containing active disinfectant outside houses, and limiting access to the site by visitors and vehicles (Fedorka-Cray et al., 2000). A study by Nollet et al. (2004) used multivariable analysis to look at risk factors for prevalence of Salmonella spp on pig farms; they found that fully slatted floors were protective compared to floors with less than 50% slatted ($p = 0.030$). The rationale is that fully slatted floors allow feces to immediately flow to the manure pit and away from the pigs, thus reducing the contact with feces (Nollet et al., 2004).

Intervention measures applied in pre-harvest production of pork is considered one of the best ways to reduce incidence of Salmonella spp in pork. The on-farm risk factors are important to identify, but lack of research has made it difficult to fully understand and evaluate risk factors and interventions. It is accepted that controlling Salmonella spp at the farm level has a major
impact by reducing the contamination pressure upon entry to the slaughterhouses and assists in increased efficiency of post-harvest contamination control measures. It is critical to identify pre-harvest interventions and controls of *Salmonella* spp in the pre-harvest pork production chain (Rostagno and Callaway, 2012). The most practical recommendation for *Salmonella* spp control on swine farms is to implement “good management practices” for disease control (Funk and Gebreyes, 2004).

*Salmonella* spp can persist in swine facilities and in swine carriers. A study of *S. Typhimurium* in pig herds showed that *S. Typhimurium* can be transmitted through pig herds. After placing an infected pig, 41% of tracer pigs were *Salmonella*-positive over 6 weeks. Artificially inoculated pigs intermittently shed *Salmonella* spp over 6 weeks. In pig facilities, it has been shown that *Salmonella* spp can persist in a paddock environment and that contaminated pastures are able to cause infections in naïve pigs. It was found that *Salmonella* spp only persisted in the soil for no longer than 5 weeks when paddocks were vacated and some huts remained contaminated for seven weeks. This highlighted the fact that facility hygiene is critical to avoid persistence of *Salmonella* spp (A. N. Jensen et al., 2006). A study examining the long-term shed of *S. Choleraesuis* found that pigs can intermittently shed for up to 5 months after infection (Anderson et al., 2000). Carrier state in pigs is difficult to detect in live animals, either by bacteriological or serological methods, and may bias pig monitoring program. *Salmonella* spp prevalence not only varies over time within a herd, but also over the lifetime of the pigs (Nollet et al., 2005).


Chickens are a major source of *Salmonella* spp exposure resulting in foodborne-illnesses in people. From 1998-2008, the two highest food commodities attributed to outbreaks caused by *Salmonella* spp were poultry (30%) and eggs (24%). Outbreaks caused by *Salmonella Enteritidis* were most attributed commonly to eggs (64%) and poultry (18%) (CDC, 2013). Infection occurs most commonly by exposure to *Salmonella* spp in fecally-contaminated litter, feed, water, fluff, dust, shavings, straw, insects, equipment, and other fomites (i.e., horizontal transmission) (Poppe, 2000). Prevention young chicks’ exposure to *Salmonella* spp is important because such infections can lead to persistent intermittent shedding and induction of carrier chickens (Van Immerseel et al., 2004).
Chickens typically are asymptomatic carriers and *Salmonella* spp can be trans-ovari ally (vertically) transmitted from the chicken to the egg as a bacterial passenger acquired as the ovum is released from the ovarian follicle and becomes sealed in the shell as the developing egg transits the oviduct. *Salmonella* serovars *S*. Pullorum and *S*. Gallinarum can be transmitted vertically, but are of little concern to humans because they are poultry-specific serovars (Poppe, 2000). *S*. Enteritidis has been shown to pass by vertical transmission; and it is postulated that *Salmonella* serovars *S*. Typimurium, *S*. Heidelberg, and *S*. Menston can also be vertically transmitted (Humphrey et al., 1989; Poppe, 2000).

Interventions include rodent control, feed mill sanitation/biosecurity, and vaccination of layer hens. Snow et al (2010) looked at risk factors for *Salmonella* spp on commercial egg-laying farms. They showed that a vaccine against *S*. Enteritidis is highly protective on egg-laying farms. Population attributable fractions found significance ($p = 0.006$ and $p = 0.000$) for the presence of mice and rats, which suggests that effective rodent control may significantly reduce the amount of *S*. Enteritidis in the population (Snow et al., 2010). In this investigation, their findings also suggested that a company feed mill was associated with an increased risk of *S*. Enteritidis infection versus farms mixing feed on the premise or using a national compouder. Prevention of horizontal transmission of *Salmonella* spp to chickens (through biosecurity measures) and vertical transmission in laying hens (through vaccination) (Snow et al., 2010)

### 1.15. *Salmonella* in Wildlife

Rodent populations may be an important source of transmission of *Salmonella* spp to many different species in food production. Wildlife and rodents are attracted to spilled feedstuffs, the availability of water, and presence of shelter, all resources which are accessible to them at most farms. Rodents acquire their infection from sources such as livestock feces, feces of other wild animals, wild birds, and/or from their own family (Meerburg and Kijlstra, 7AD). Contamination of environmental sources on farms with *Salmonella* spp might include rodents (Meerburg and Kijlstra, 7AD; Snow et al., 2010). It has been established in experimentally infected rodents that there can be a persistent chronic infection and *Salmonella* spp can be recovered from systemic sites in asymptomatic mice up to one year after infection. (Lawley et al., 2008; Monack et al., 2004). *S*. Typhimurium can be transmitted to naïve mice housed in the same cage as mice persistently infected with *S*. Typhimurium (Lawley et al., 2008).
Wild birds may also be a contributing factor with *Salmonella* contamination. *Salmonella* spp have been found in healthy wild birds captured near dairies, but low prevalence of *Salmonella* spp makes wild birds an unlikely vector or reservoir of *Salmonella* spp (Kirk et al., 2002). Livestock feed stored on the ground is susceptible to contamination by rodent and bird feces. Livestock ingesting feed contaminated by wildlife feces is a significant potential route of *Salmonella* spp infection to livestock (Daniels et al., 2003).

### 1.16. *Salmonella* in Horses

Equine salmonellosis is most commonly observed on breeding farms and in veterinary hospitals. Common themes of *Salmonella* spp outbreaks in adult horses are congregation of animals and alteration of gastrointestinal function by feed and/or water deprivation, disease or medication. Anorexia, antimicrobial administration, intestinal surgery and marked changes in diet increase the susceptibility of horses to *Salmonella* spp. Foals are more susceptible than adult horses due to their incompletely developed immune system and the lack of competitive gut flora (Ernst et al., 2004).

In clinically normal horses, an investigation of fecal shedding of *Salmonella* spp by horses in the United States showed a very low prevalence of only 0.8% (Traub-Dargatz et al., 2000). Another study examined fecal shedding detected by PCR in competing endurance horses and found only 0.5% of fecal samples tested positive for *Salmonella* spp (Fielding et al., 2013). A similar pilot study looked at the effect of transportation, environmental changes, and athletic competition on fecal shedding of *Salmonella* spp in sport horses. Sampling of 20 horses with a total of 125 samples, found all fecal samples were negative (0% positive) for *Salmonella* spp (Taintor et al., 2014). It appears that healthy competition horses do not commonly shed *Salmonella* spp and therefore are at low risk for transmission between horses.

In contrast to healthy horses, the prevalence of *Salmonella* spp in horses that have been hospitalized because of acute gastrointestinal tract disease or colic ranges from 3% to 13% (Ernst et al., 2004; Kim et al., 2001). Risk factors for horses with gastrointestinal disease associated with *Salmonella*-positive fecal shedding include transportation distance (travel time >1 hour), abnormal findings on nasogastric intubation, diarrhea, leukopenia (5000 white blood cells/mL), previous antimicrobial therapy, abdominal surgery, and duration of hospitalization (Burgess and Morley, 2014a).
Veterinary hospitals are required to treat horses with salmonellosis and concurrently house high-risk patients. Horses with colic that undergo abdominal surgery suffer significant stress. A horse’s large colon may be emptied, feed is often withheld or they are treated with antimicrobials, undergo anesthesia and experience different degrees of ileus. All of these events contribute to producing a patient that is extremely susceptible to developing salmonellosis when exposed to even very small numbers of Salmonella spp (House and Smith, 2000). For all these reasons, an effective infectious disease-control program is essential.

At many veterinary hospitals, horses are isolated if they have clinical signs (fever, leucopenia, and diarrhea) compatible with salmonellosis until the confirmation of diagnosis for Salmonella spp. Clinically affected patients produce large volumes of diarrhea which rapidly amplify environmental contamination (House and Smith, 2000). In areas with infected horses, Salmonella spp can be isolated from the drains, floors, dust and crevices in stall walls (House and Smith, 2000). Animal care providers can easily carry the organism from one horse to another on clothing and hands, and contaminated equipment has also been implicated as a vector in past veterinary hospital outbreaks. Biosafety protocols exist to minimize Salmonella spp environmental contamination and the risk of exposing susceptible animals and people to Salmonella spp. These protocols include isolation of infected animals, steaming or burning of their manure and bedding to kill Salmonella spp, management of feed and water to minimize Salmonella contamination, chlorination of water, fly and rodent control and personnel training. It is critical to monitor environmental Salmonella contamination and the incidence of Salmonella spp shedding in horses at risk in order to assess the effectiveness of and to recognize deficiencies in Salmonella spp control strategies (Burgess and Morley, 2014a). Many times other livestock introduce pathogenic Salmonella spp into veterinary hospitals. So from the perspective of Salmonella spp control, it is encouraged to hospitalize horses in facilities separate from those of other livestock (House and Smith, 2000).

1.17. Salmonella Outbreaks in Veterinary Teaching Hospitals

1.17.1. Michigan State University – Large Animal Clinic; 1996

A multidrug-resistant S. Typhimurium was identified as the etiologic agent responsible for an outbreak of nosocomial salmonellosis involving 28 hospitalized horses at Michigan State University’s Large Animal Clinic from May 1996 through discharge of the last affected horse in February 1997. The outbreak started May 2, 1996, with the admission of the point-source foal...
and ended January 29, 1997, with the discharge of the last horse documented to have nosocomial salmonellosis. This outbreak had a fatality rate of 44% (8/28 infected horses).

Over the 9-month period, 10 pulsed-field gel electrophoresis (PFGE) patterns were documented: 1a, 1b, 1c, 1d, 1e, 2a, 2b, 2c, 2d, 2e. Three PFGE patterns (1a, 1e, and 2a) were identified for the S. Typhimurium isolate recovered from the point-source foal. This is the first report to document changes in PFGE patterns during the natural course of an outbreak of salmonellosis in horses. Pattern changes were evident between initial isolates and subsequent isolates recovered from 5 horses and all change was explained by 1 or 2 genetic events (Schott et al., 2001).

The organism that caused this outbreak was able to persist in the environment despite hospital closure for extensive cleaning and disinfection. During hospital closure and disinfection, PCR testing of environmental samples yielded positive results for 28 of 237 (12%) samples collected from several areas of the hospital and the isolation facility after cleaning and disinfection. In contrast, the multidrug-resistant S. Typhimurium was still viable and culture-positive in 3 of 241 environmental samples.

Horses that developed nosocomial salmonellosis had been placed in 1 of 2 stalls. Repeated PCR testing suggested that Salmonella organisms had survived in vertical drainpipes. An added cleaning protocol for all vacated isolation stalls was implemented where drain plugs were installed at the top of the vertical pipes, and the drain bowl left filled with a concentrated disinfectant solution (Schott et al., 2001).

The mode of transmission for the two initially affected horses admitted on the same evening as the point-source filly was attributed to contamination of hospital personnel and shared equipment spreading the organism. The organism persisted in the environment after more stringent stall and ward cleaning. The control of this outbreak was likely the result of several factors. First, hospital closure and disinfection helped to slow the outbreak. Second, institution of stricter criteria for isolation of hospitalized patients shedding Salmonella spp helped contain the organism and prevented wider spread through the hospital. Third, other modifications to infection control policies that were instituted likely helped control the spread of the organism. Finally, the decrease in new nosocomial infections paralleled decreases in caseload and ambient temperature during the winter months (Schott et al., 2001).
After the outbreak, outpatient stalls were limited to use by 1 horse daily. These stalls were hose cleaned, doused with bleach, and allowed to air-dry overnight. The floors of examination rooms, treatment rooms, hospital aisles, and ward aisles were hosed daily and disinfected twice weekly with a phenolic compound using a spray applicator. Each hospital ward was closed 1 or more times annually for complete cleaning and disinfection. Environmental samples were regularly collected from sites throughout the hospital and submitted for bacterial culture for *Salmonella* spp. One member of the technical staff was designated as the infection control officer with responsibilities that included regular collection of environmental samples for bacterial culture, release of restricted stalls, and maintenance of a database for results of bacterial culture of fecal and environmental samples and the schedule for regular cleaning and disinfection of hospital areas. A summary of results of bacterial culture of fecal samples detailing the frequency at which *Salmonella* spp were isolated and information about clinical cases of salmonellosis was distributed regularly to large animal hospital faculty, residents, technicians, and hospital staff (Schott et al., 2001).

### 1.17.2. Purdue University – Teaching Hospital; 1999 – 2000

An outbreak of salmonellosis from a multidrug-resistant *S*. Typhimurium occurred in hospitalized horses at Purdue University teaching hospital. The outbreak started in August 1999, with the admission of a colic case shedding the multidrug-resistant *S*. Typhimurium which was euthanized due to deteriorating conditions that same day. In a retrospective evaluation of isolates recovered from hospital cases, a small and unrecognized outbreak of multidrug-resistant *S*. Typhimurium had occurred between August and December 1999. This smaller, unrecognized cluster was followed by a large cluster of clinical cases of salmonellosis from April to June, 2000. The hospital was closed for a 10-week period after an unusual increase in the number of horses with diarrhea and suspected salmonellosis. The outbreak had a fatality rate of 42% (14/33). After the closure of the hospital, it was extensively cleaned and disinfected. Throughout the hospital’s closure, approximately 1100 environmental samples were taken from areas such as stalls, drains, common areas (ward aisles and breezeways), the neonatal intensive care unit, treatment rooms, operating theatres, radiology, laundry, garage, farriery, treadmill facilities, machinery, locker rooms, bathrooms, reception area, and office. Enhancements such as improved rodent and cockroach control, repair of ceiling leaks, and repair of areas of lifting floor material were carried out in the hospital (Ward et al., 2005b).
The hospital was reopened in August, 2000. *Salmonella* was only isolated from 12 sites in the hospital. Eighteen of the 33 cases in this outbreak had surgical procedures performed on them, and it is possible that some cases were infected during surgery. Immediately following the outbreak, disease control policy was revised to include semi-annual sampling of the hospital environment for *Salmonella* contamination. The three-month gap during the outbreak suggests that *Salmonella* spp are able to persist in the hospital environment until ambient temperatures increased and more clinical cases were observed. The endemic hospital strain of *S. Typhimurium* responsible for the outbreak was identified by serotyping, antibiogram, phage typing and PFGE. The authors of the investigation suggest that patients presenting at teaching hospitals with gastrointestinal disease should be carefully evaluated on admission for salmonellosis, and monitored for *Salmonella* spp shedding during hospitalization. In addition, the authors suggest that active environmental monitoring is necessary to identify sources of *Salmonella* contamination (Ward et al., 2005b).

### 1.17.3. University of Pennsylvania School of Veterinary Medicine – New Bolton Center; 2003 – 2005

An outbreak of salmonellosis at the University of Pennsylvania School of Veterinary Medicine’s New Bolton Center occurred from July 2003 to January 2005. This nosocomial outbreak was caused by *S. Newport* multidrug resistant (MDR)-AmpC, which is distinguished by presence of the plasmid-mediated *ampC* gene (*bla*CMY2) that encodes resistance to extended-spectrum cephalosporins. The outbreak occurred despite having an Infection Control Program (ICP) in place, was characterized by a high infection and case fatality rate, and had substantial financial impact. The outbreak affected 11% of patients (54 equine; 7 non-equine) of 554 total animals and had an all-species case fatality rate of 36.1% (approximately 1 in 3). The outbreak attack rates for equine patients was 2.1% and for non-equine patients was 1.0%. The case fatality rate in horses was 31.5% (17/54) (Schaer et al., 2010).

The index case was identified retrospectively to be a 3-year-old thoroughbred racehorse admitted on July 1, 2003 for colic, but the horse was not identified as culture positive for *Salmonella* until July 10, 2003. In February 2004, in response to a perceived increase in salmonellosis, a retrospective and prospective epidemiological investigation was initiated. Historical PFGE and PCR (detection of *bla*CMY and *bla*TEM genes) tests were performed to confirm genetic homogeneity of strains isolated during the outbreak. During a 35-week period
(July 2003–February 2004), patients positive for serotypes Newport (n=28) and Typhimurium (n=13) were identified. Over the 10-week period from March 1 until hospital closure (May 10, 2004), isolates identified as S. Newport MDR-AmpC were recovered from 33 patients and only 2 patients were positive for S. Typhimurium. PFGE data indicating genetic homogeneity among Newport isolates provided definitive evidence of an outbreak. A staged re-opening of the hospital began in August, 2004, but the hospital was not fully operational until January 2005 when the intensive care unit and neonatal intensive care unit were reopened (Schaer et al., 2010).

Before the outbreak, an original environmental surveillance (ORIG SURV) program had been performed as outlined in the existing ICP. Five individual samples were collected every 2 weeks from 1 of 5 predetermined high-traffic locations in the hospital that included selected barn locations, postmortem room, patient admission area, and surgery areas. At the quarterly Quality Assurance Committee meetings or when the Infectious Disease Committee met (convened as needed), results were reviewed. The sampling procedure involved using a dry sterile gauze sponge to swab a dry surface. After selenite broth enrichment and differential plating techniques, identified Salmonella colonies were tested for antimicrobial susceptibility, and serogrouping, serotyping, and PFGE analyses were performed. Samples were stored in the Salmonella Reference Center for further analysis (Schaer et al., 2010).

Following consultation with a biosecurity specialist 2 weeks prior to hospital closure, environmental sample collection and culture methods were changed to a modified environmental surveillance (MOD SURV). Enhanced sample collection with an electrostatic wipe and sweeper passed over a broad surface within a sample area and areas were divided into hand and foot samples. Samples were processed by a multistage procedure involving pre-enrichment, enrichment, and differential plating designed to select for Salmonella spp. Presumptive Salmonella colonies were identified, antimicrobial susceptibilities determined, and serogroup, serotype, PFGE, and PCR assays were performed. This method had financial and administrative restrictions that initially limited the number of samples collected and analyzed. A total of 120 samples were collected to determine the initial extent of hospital contamination. After closure, to determine the extent of contamination throughout the facility an additional 222 samples were collected. More than 1,100 samples were submitted and processed to ensure appropriate decontamination and remediation throughout the clean-up process (Schaer et al., 2010).
With the ORIG SURV, 722 samples were collected between January 1998 and March 3, 2004 with a total of 3.3% (24) being positive for *Salmonella* spp; 10 were serogroup C2 (*S. Newport* is a group C2 serovar), 7 serogroup B, and 1 was serogroup D. After the index case was admitted, 41.7% (10) of the samples were positive; 7 were serogroup C2, 3 were serogroup B. In March, 2004, a total of 171 samples were collected in areas of the hospital where salmonellosis had occurred with 8.8% (15) of samples collected from the Intensive Care Unit and the Neonatal Intensive Care Unit (ICU/NICU) area and orthopedic barn testing positive. After these areas were depopulated, extensively cleaned, decontaminated, and the facility underwent remediation, ORIG SURV environmental samples collected were negative. Despite negative environmental samples from extensive cleaning and decontamination, 5 additional cases of salmonellosis occurred in these areas afterwards (Schaer et al., 2010).

The 120 MOD SURV samples showed widespread environmental contamination with 30% (36/120) being positive for *S. Newport* MDR-*AmpC*. Confirmation of extensive and repeated contamination of the human traffic and animal areas, combined with findings from a site visit by biosecurity experts, resulted in full hospital closure. To determine contamination across the extended campus after closure, 222 samples were collected and 3 were positive for unrelated *Salmonella* spp. Certain functions confined to non-contaminated areas of the campus operated normally. The school's ambulatory and outpatient services were relocated to non-restricted zones. Physical barriers, foot baths and mats, and temporary fences were placed to control traffic and prevent further contamination during cleaning and decontamination (Schaer et al., 2010).

An interim Director of Biosecurity was appointed after hospital closure to manage facility remediation and decontamination. All animal housing and clinical spaces were subjected to a 3 or 4 stage cleaning and disinfection protocol by faculty members, house officers, and hospital staff. Extensive remediation to repair damaged surfaces included sandblasting and resurfacing, cleaning, disinfection, and repainting of the 4 main cement-block barns. All dirt stall flooring was removed and replaced with concrete. Stall mats were removed and a polyurethane-based monolithic flooring system was installed in all stalls and animal handling areas. Remediation of human traffic areas within clinical spaces included removing damaged equipment and replacing non-cleanable surfaces. The ICU/NICU was decontaminated by professional contractors with a chlorine dioxide gas-phase decontaminant (Schaer et al., 2010).
The total financial impact of the outbreak and subsequent closure was estimated to be $4.12 million. The estimated lost revenue caused by closure, staged reopening, and decreased caseload was $3.25 million, $704,574 was the cost of facility remediation and decontamination and $160,492 was revenue lost because of hospital coverage of patient bills. The monthly revenue did not return to previously generated income until April 2005 (Schaer et al., 2010).

After the hospital returned to full operation, a full commitment to biosecurity was made at the highest level of the school. A long-term biosecurity plan was developed by a new Director of Biosecurity with the assistance of a Biosecurity Advisory Committee that included representatives from all clinical services, critical support staff, and diagnostic services. Dedicated faculty and staff positions with sole responsibility of infection control were created (Director of Biosecurity, Biosecurity Assistant). A modified ICP was implemented which incorporated sampling of all inpatients throughout hospitalization and extensive environmental surveillance, risk stratification, and traffic control of patients and people, adherence to excellent hygiene practices, and stakeholder education. Rigorous fecal surveillance of all inpatients was added to the revised ICP with the aim of establishing incidence of *Salmonella* shedding in various segments of the hospital’s patient population. A more rigorous patient algorithm triggered a mandatory series of 3 fecal cultures, implementation of barrier precautions, and strict patient relocation to isolation if specific criteria are met. Clinical status of patients in high risk areas is monitored by the biosecurity staff in liaison with attending clinicians (Schaer et al., 2010).

This nosocomial outbreak occurred despite an existing ICP. Failures of the original ICP included lack of a dedicated individual responsible for biosecurity, reliance on passive and less than comprehensive patient surveillance (active and algorithm-driven components), poor enforcement of and compliance with existing protocols, no centralized database for analysis of patient or environmental results, no real-time data evaluation, and no modifications to the program in response to changes in microbial threats. Patients were not housed based on risk for nosocomial infection and there were few explicit directives for implementation of barrier precautions in areas other than isolation. There was no definition of what constituted nosocomial disease in the previous ICP. A passive approach to patient surveillance in place at the time of the outbreak was based on a loosely followed patient algorithm that targeted animals with diarrhea only. No routine surveillance of healthy or at risk patients was performed. Gastrointestinal cases were routinely triaged to a barn housing outpatients and patients hospitalized for elective
procedures that further compounded the contamination. The ORIG SURV was flawed and led to the false belief that the hospital was not contaminated. The MOD SURV technique was essential in determining the degree of hospital contamination (Schaer et al., 2010).

1.17.4. Cornell University – Equine and Farm Animal Hospital

An outbreak of salmonellosis at Cornell University’s Equine and Farm Animal Hospital (EFAH) occurred from August 2006 through August 2008. This nosocomial outbreak was caused by S. Oranienburg. PFGE analysis was used to determine genetic homogeneity among S. Oranienburg isolates. The index patient was an 8-year-old mixed breed mare with chronic infection and drainage from the right hind sole (Cummings et al., 2014).

S. Oranienburg was identified in 28 EFAH patients between January 1, 2006, and June 1, 2011. Retrospective analysis determined that 27 additional patients were fecal culture positive for S. Oranienburg: 19 horses, 5 alpacas, and 3 cows. The last patient known to be positive for S. Oranienburg was admitted January, 2008. Only 70% (19/27) of these cases were nosocomial infections, while 15% (4/27) originated from farms that were regular clients of the EFAH and that had been the source of multiple admissions to the hospital following the index case, 4% (1/27) were treated at a private referral hospital (approximately 100 miles away) that frequently referred patients to the EFAH, and 11% (3/27) were unrelated community-acquired infections not epidemiologically linked to the EFAH. Multiple PFGE types isolated from one animal indicated that this animal was co-infected by multiple circulating S. Oranienburg subtypes or reinfection by closely related strains. The mechanism for the S. Oranienburg diversification over the course of the outbreak was not identified, but could have been due to the loss or acquisition of mobile genetic elements, nonhomologous recombination events, chromosomal rearrangements, or point mutations. The rapid diversification of S. Oranienburg during the outbreak is suggested as the most likely explanation for the observed range of PFGE types (Cummings et al., 2014).

A total of 1182 environmental samples were collected during the 2006–2008 outbreak period and 0.5% were positive for S. Oranienburg. Positive samples were from two isolation stalls, the floor of the intensive care unit, the floor of the cattle barn, an equipment cart, and a rope used with downer cows. The environmental contamination could be attributed to the index patient being walked repeatedly throughout the hospital (radiology and surgery areas) prior to receipt of culture results (Cummings et al., 2014).
This study highlights the risk of *Salmonella* transmission from hospitalized patients to herd mates following discharge. This study provides evidence that animals can became infected on their home farms via transmission from herd mates that had recently visited a veterinary hospital during an outbreak. Heightened biosecurity efforts are attributed to the cessation of the outbreak. Biosecurity efforts included biosecurity training sessions for hospital personnel, additional restrictions on human traffic, disinfectant footbaths in front of every stall and at the entrance to each hospital ward, an increase in hand disinfection stations throughout the hospital, twice weekly fecal *Salmonella* cultures performed on all hospitalized patients, and fecal *Salmonella* cultures performed on all outpatients. An email list was also established to notify the hospital director, biosecurity committee, clinicians, technicians, and barn manager of any suspect *Salmonella* patients before the official laboratory report was confirmed. The *Salmonella* suspect patient is immediately placed in temporary isolation until final culture results are confirmed. Temporary isolation involves limited access to patients, the application of Tyvek coveralls, exam gloves, and plastic boots prior to entering the stall, with removal and disposal upon exiting. Floors throughout the hospital are disinfected at least twice daily with an industrial walk-behind scrubber. PFGE subtyping is now performed at the time of *Salmonella* spp isolation from fecal and environmental samples to facilitate assessment of epidemiologic relatedness (Cummings et al., 2014).

1.18. *Salmonella* Biosecurity Efforts

Biosecurity refers to all hygienic practices designed to prevent occurrences of infectious diseases that include preventing the introduction of infectious agents, controlling their spread within populations or facilities, and containment or disinfection of infectious materials (Morley, 2002). Nosocomial infection outbreaks can be very costly with hospital closures, lost revenue, facility remediations and decontaminations, coverage of patient bills, and mitigation costs as well as the loss of public confidence in a hospital (Burgess and Morley, 2014a; Dargatz and Traub-Dargatz, 2004; Kurowski et al., 2002; Morley, 2002; Patterson et al., 2005; Schaer et al., 2010). A total of 82% of veterinary teaching hospitals reported outbreaks of nosocomial infection between 2002 to 2007 with 50% of the veterinary teaching hospitals reporting that zoonotic infections had occurred (Benedict et al., 2008). The most commonly associated agent responsible for nosocomial outbreaks in both small and large animal veterinary teaching hospitals is *Salmonella* (Benedict et al., 2008; Cherry et al., 2004; Morley, 2002; Schott et al., 2001;
Steneroden et al., 2010). The case definition for nosocomial *Salmonella* infection is defined as a salmonellosis infection identified after animals have been hospitalized for 72 hours or longer and when the serotype and antimicrobial susceptibility pattern of *Salmonella* isolates from the primary and nosocomial cases are the same (Ekiri et al., 2010). Case definitions can vary depending on the study. One study by Ekiri et al (2009) identified nosocomial cases where patients had tested negative for *Salmonella* spp in samples obtained at the time of admission and tested positive 48 hours after admission, and the primary case had a positive result for *Salmonella* spp in samples obtained at the time of admission, and shared the same serotype and antibiogram as the isolate from the nosocomial case, and overlap between admission and discharge dates of the primary and nosocomial cases. When environmental contamination was attributed as the source of infection, the case was never exposed to the primary case and the case had negative results for *Salmonella* spp in samples obtained at the time of admission, but tested positive afterward for a *Salmonella* that was the same serotype and antibiogram profile as a *Salmonella*-positive environmental sample collected during the period of hospitalization (Ekiri et al., 2009).

The environment can be a potential source and reservoir for the transmission of *Salmonella* spp in a veterinary hospital. Persistence of *Salmonella* spp in the environment has been implicated for the spread of *Salmonella* spp among patients in many nosocomial outbreaks of salmonellosis (Amavisit et al., 2001; Castor et al., 1989; Dunowska et al., 2007; Schaer et al., 2010; Schott et al., 2001; Ward et al., 2005b). Most veterinary hospitals’ surveillance programs involve sampling patients that are a high risk of shedding *Salmonella* spp at admission, which involves sampling patients with a gastrointestinal tract disease such as colic, diarrhea and patients with fever and leucopenia (Morley, 2002; Schott et al., 2001; Ward et al., 2005a). Some university veterinary hospitals may utilize environmental sampling as part of their surveillance for infection control programs (Burgess et al., 2004; Burgess and Morley, 2014b; Dunowska et al., 2007; Schott et al., 2001; Traub-Dargatz et al., 2004).

The “gold standard” in detection of *Salmonella* spp in feces is a standard fecal culture. This requires several days for results and is limited in sensitivity, although enrichment techniques and the use of serial culture methods improve the sensitivity of culture. The fecal culture has advantages in that only viable organisms are identified and cultured organisms can be assessed for anti-microbial susceptibility (McKenzie and Hodgson, 2011). Utilizing PCR for the
rapid detection of *Salmonella* spp can be an alternative to conventional culture methods for surveillance and has the potential to reduce the risk of nosocomial infections through the provision of highly accurate and rapid pathogen detection (Pusterla et al., 2010). PCR analysis for detection of *Salmonella* spp is fast and inexpensive, but may detect non-viable organisms or may have poor PCR specificity with the possibility of false-positives (McKenzie and Hodgson, 2011). If both procedures are carried out on samples and both are negative, it can reasonably be assumed that a patient is not a carrier or that the environment is clear of *Salmonella* (Pusterla et al., 2010).

Sites of environmental samples should not be selected at random. Environment sample sites should be areas considered at high risk of contamination. Routine environmental sampling consisting of 25 of 100 hospital sites targeted for sampling should be sufficient to detect (with 95% confidence) one or more sites with evidence of *Salmonella* contamination. The prevalence of contamination expected during an outbreak among the 100 sites would be 10% or higher (Ekiri et al., 2010). Environmental *Salmonella* contamination of veterinary hospitals poses a risk of nosocomial infection to patients and the risk of zoonotic infections to clients, clinicians, technicians, and students (Ewart et al., 2001; Pandya et al., 2009). However, without input from surveillance data, infection control practices are likely to be guided more by emotions and opinions than by data and evidence. Every veterinary hospital should develop a surveillance and infection control program for control of nosocomial infections that is tailored to fit the needs of the operation and the personnel (Morley, 2004). In order to control important nosocomial hazards effectively, systematic reviews such as Hazard Analysis and Critical Control Points determination are needed (Morley, 2002). Common surveillance strategies included summarizing contagious disease diagnoses reported by clinicians and compiling results of cultures or tests performed for diagnostic purposes (Benedict et al., 2008). This would be a passive approach to *Salmonella* surveillance because the surveillance might be conducted by using fecal samples submitted for other purposes and data collected through medical records or billing data (Morley, 2004). Institutions with passive surveillance typically conduct surveillance at irregular intervals that is triggered by a reported perceived risk in the hospital (Benedict et al., 2008). An active approach to *Salmonella* surveillance might be collection of fecal specimens from all or a subset of the hospital population and data collection by means tailored to the effort (Morley, 2004). Active surveillance strategies included routinely submitting environmental samples for bacterial
culture and collecting samples from patients to detect specific contagious pathogens. Even though there may be differences among infection control programs, which aspects of these programs are the most important or have the greatest impact on the risk of nosocomial infection in these hospitals has not been studied (Benedict et al., 2008).

A survey of veterinary teaching hospitals found that 89% of locations have biosecurity protocols, but only 42% require personnel to complete a biosecurity training program (Benedict et al., 2008). The success of the biosecurity programs is greatly dependent on participation and compliance of all personnel working in the veterinary teaching hospitals. A common barrier to compliance is the lack of appropriate resources or facilities and a lack of appropriate motivation among personnel. To achieve good compliance from personnel, effective communication of what is expected as well as why these procedures are important is essential. Compliance generally degrades over time with less convenient procedures and when personnel are not trained to be fully aware or appreciative of goals and consequences. Surveillance should be conducted to ensure compliance and that protocols remain effective in the ever-changing environment of a veterinary hospital (Burgess and Morley, 2014b). Periodic training and orientations on surveillance and infection control practices to new hospital personnel, faculty, residents, interns, and DVM students introduces and reinforces the concepts of biosecurity and biocontainment (Ekiri et al., 2010; Traub-Dargatz et al., 2004).

Many institutions were reportedly engaged in active or passive surveillance for infectious diseases, but these were not necessarily conducted at predetermined temporal intervals (Benedict et al., 2008). Preventive measures that can be used to decrease infectious disease transmission risk include environmental and personal hygiene such as rigorous hand hygiene (Burgess and Morley, 2014b; Milton et al., 2015; Shea and Shaw, 2012). Hygiene practices of hospital personnel can be addressed through regular training on biosecurity, providing ready access to handwashing stations as well as dispensers of hand sanitizers and strategically placed footbaths and having special clothing readily available to facilitate barrier precautions (Traub-Dargatz et al., 2004). Veterinarians and clients recognize the value of surveillance and infection control practices to reduce the risk of hospital-acquired infections and that the current cost is reasonable. Most referring veterinarians and clients indicate testing patients for *Salmonella* spp at admission and during hospitalization is justified (Ekiri et al., 2014). The AVMA provides accredited institutions with general expectations regarding maintaining clean facilities and equipment in the
veterinary teaching hospitals, but no specifications are outlined in reference to a certain level of infection control or biosecurity (Benedict et al., 2008). Appropriate infection control plans must be tailored to the individual facility, but a critical component of any effective infection control program is educating personnel about potential hazards and the value of established control measures, and a surveillance component for collecting information necessary to guide ongoing efforts (Benedict et al., 2008; Morley, 2004; Traub-Dargatz et al., 2004).

1.19. Introduction

Salmonella spp infections are the leading cause of human foodborne illness among laboratory confirmed bacterial foodborne pathogens (Huang et al., 2016; Scallan et al., 2011). It appears that the 2020 national health objective target will not be met because the incidence rate of non-typhoidal Salmonella spp infections remains unchanged from the 2006-2008 rates (Centers for Disease Control and Prevention (CDC), 2011; Huang et al., 2016). Interventions can be applied pre-harvest, post-harvest, or both to reduce incidence of Salmonella in foods (Rostagno and Callaway, 2012). Animals carrying Salmonella spp when leaving the production farm constitute the original source of Salmonella spp in food. Therefore, a critical control point is reduction of the amount of Salmonella spp organisms entering the processing plant (Cox and Pavic, 2010). Pre-harvest interventions at the farm level are critical to reduce the Salmonella spp contamination pressure entering abattoirs (Rostagno and Callaway, 2012). Interventions already in use include biosecurity, vaccination, competitive exclusion, prebiotics and probiotics, feed and water control, and experimental interventions (Cox and Pavic, 2010). Sub-therapeutic levels of antibiotics have been routinely fed to livestock, but the emergence of infectious diseases caused by drug-resistant bacteria has stimulated the search for alternatives to conventional antibiotics (Jassim and Limoges, 2014).

The reduction of foodborne pathogens requires a comprehensive intervention program at the farm before shipment to processing plants. One potential pre-harvest intervention to reduce sources of foodborne pathogen contamination would be the use of specific phages to selectively reduce or eliminate susceptible bacteria from designated environments and animals (Jassim and Limoges, 2014). Bacteriophages can address food safety concerns without residual effects of antibiotics in animal meat, meat products, and milk; they also have therapeutic potential in treating bacterial infections in animals and can prevent fatal infections (Tiwari et al., 2014). It also has been shown that there is little danger of susceptible animals contracting disease from
infected animals with which they were in contact or from premises in which they had been kept, when the infected animals have been treated with phage (Smith and Huggins, 1983). Bactericidal phages may provide a feasible natural, nontoxic approach for controlling several human pathogens in livestock (Jassim and Limoges, 2014).

A major drawbacks of phage therapy, is the possible emergence of phage-resistant derivatives. Any phage to be applied commercially in the control of foodborne pathogens should be examined for its safety in the animal and for its tendency to produce phage-resistant bacterial mutants (Mahony et al., 2011).

The fitness cost of phage-resistant bacterial strains is particularly high and may cost the bacterium in virulence reduction (León and Bastías, 2015). Some phage-resistant mutants have been shown to be avirulent such as a phage-resistant mutant of S. Enteritidis in a Caenorhabditis elegans animal model (Santander and Robeson, 2007). Decreased virulence has also been seen in phage-resistant mutants of Flavobacterium columnare in a zebrafish model, phage-resistant mutants of Vibrio cholerae in mice, and phage-resistant mutants of Staphylococcus aureus in mice (Capparelli et al., 2010b; Laanto et al., 2012; Shamim Hasan Zahid et al., 2008). Phage-resistant strains which have been reported to have reduced survival in vivo include phage-resistant strains of Esherichia coli and Campylobacter jejuni (Scott et al., 2007; Smith and Huggins, 1983). From epidemiological and environmental observations of Vibrio cholerae, the predation of phage on the bacterial host and the decreased fitness of phage-resistant Vibrio cholerae provides an explanation for the cyclically epidemic cycles of cholera outbreaks (M. A Jensen et al., 2006). Immunization with live and heat killed avirulent phage-resistant mutants of Staphylococcus aureus have been shown to confer broad immunity against staphylococcal infections in mice (Capparelli et al., 2010b). Another study showed that phage-resistance in S. Paratyphi B reduced virulence and is an excellent live vaccine candidate due to the protection it confers from infections of S. Paratyphi B, S. Dublin, S. Typhimurium, and S. Virchow (Capparelli et al., 2010a).

These studies, however, do not exclude the chance of phage-resistance occurring concomitantly with increased virulence to a pathogen. Many virulence factors are bacteriophage-encoded and if a hypothetical toxin-carrying temperate phage were to integrate into the DNA of a Salmonella bacterium, and the newly acquired prophage confers resistance to infection by
super-infection exclusion, then the phage-resistance can increase virulence (Boyd and Brüssow, 2002; Labrie et al., 2010; Loc-Carrillo and Abedon, 2011; Miao and Miller, 1999).

In order for a phage treatment to reduce foodborne pathogens in animals, the effect of phage-resistance on virulence of the pathogen should be determined. As part of this thesis project, a phage-resistant S. Newport strain was generated and inoculated into calves along with the parent strain to test the hypothesis that phage-resistance resulted in attenuation of the pathogen.

Environmental Salmonella contamination poses a risk to livestock for acquiring Salmonella spp (Ewart et al., 2001; Pandya et al., 2009). Biosecurity and risk factor analysis of Salmonella can help determine areas of intervention to control Salmonella on farms (Fossler et al., 2005; Funk and Gebreyes, 2004). Environmental and animal samples can easily be collected in veterinary teaching hospitals (Pandya et al., 2009). Horses, ruminant livestock species, poultry, wild birds, and rodents can be reservoirs for transmitting Salmonella spp to other animals and to humans (Schott et al., 2001). Preventing and controlling potential nosocomial Salmonella infections is an important biosecurity concern. The second portion of this thesis hypothesizes that environmental sampling could identify sources or factors that contribute to the prevalence of Salmonella spp in the environment. Interventions developed to control the spread of Salmonella spp on the vet school campus might translate to develop intervention strategies for on-farm contamination issues.

1.20. References


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Chapter 2

A Study of a Bacteriophage-Resistant *Salmonella* Mutant and Infection in Calves

2.1. Abstract

Bacteriophage (phage) treatment for the reduction of multiple drug resistant *Salmonella* Newport in dairy calves is being examined in our laboratory for its clinical disease and food safety implications. An unintended consequence of phage treatment could be the emergence of fully virulent but phage resistant *Salmonella*. Working from the hypothesis that phage resistance would attenuate virulence in *S*. Newport, we generated a spontaneous mutant resistant to 4 out of 5 lytic phages used in our treatment regimen. Two pairs of 8-10 week old Holstein bull calves were challenged orally with the phage-resistant mutant. One pair was administered a dose of \(7.45 \times 10^9\) colony forming units (CFU’s) and the second pair \(1.47 \times 10^{10}\) CFU’s. A third pair of calves was challenged with a total dose of \(1.96 \times 10^{10}\) CFU’s composed of a 1:1.3 ratio of *S*. Newport parent: mutant in a competition assay designed to determine how well the mutant competed against the parent strain *in vivo*. The four calves inoculated with the phage resistant *S*. Newport mutant strain showed much reduced *Salmonella* fecal shedding and no clinical disease signs compared to the parent strain. The calves in the competition assay showed severe diarrhea and high fecal shedding of both parent and mutant *Salmonella* strains, along with signs of fever and lethargy. The decreased shedding and absence of clinical signs in the calves which received only the phage resistant strain indicates that the spontaneous generation of multi-phage resistance in *S*. Newport had an attenuating effect on virulence, perhaps by decreasing the mutant’s fitness and/or its ability to colonize its host. The results of the competition experiment suggest that the dual inoculation with parent and mutant strain restored virulence to the mutant strain and may increase fitness in the parent strain. Future experiments designed to examine the mechanism of phage resistance in this attenuated *S*. Newport mutant are planned.

2.2. Introduction

*Salmonella* species (spp) infection is the leading laboratory-confirmed cause of foodborne illness among bacterial foodborne pathogens (Huang et al., 2016; Scallan et al., 2011).
It appears that the incidence rate of non-typhoidal *Salmonella* spp infections is unchanged from the 2006-2008 rates; without progress to reduce the number of incident cases, the 2020 national health objective target may not be achievable (Centers for Disease Control and Prevention (CDC), 2011; Huang et al., 2016). Livestock and poultry infected with *Salmonella* spp at the time of transport from the production farm to the slaughter facility constitute the original or pre-harvest source of *Salmonella* spp in food. In order to reduce incidence of *Salmonella* in foods, interventions can be applied pre-harvest, post-harvest, or both. Reduction of *Salmonella* spp at the farm level by pre-harvest interventions is ideal, and constitutes a critical point for reduction of the enteropathogen contamination load entering abattoirs and processing plants; it less directly assists post-harvest intervention efforts (Rostagno and Callaway, 2012). Pre-harvest interventions include optimizing the grow-out environment, biosecurity protocols including exclusion of vectors and fomites; vaccination regimens, use of competitive exclusion (prebiotics and probiotics), feed and water control, and experimental interventions (Cox and Pavic, 2010). Sub-therapeutic levels of antibiotics have in the past been routinely fed to livestock for growth promotion and feed efficiency, but such use with increasing evidence of the emergence of drug-resistant strains of bacteria requires the development of alternatives to conventional antibiotics (Jassim and Limoges, 2014).

*Salmonella* Newport is an important pathogen found in cattle feces that is transmitted through the food chain to humans. Between 1973 and 2011, *S*. Newport was the second most common serovar linked to outbreaks of beef-attributed human salmonellosis and is responsible for more illnesses and hospitalizations than any other serovar linked to beef consumption. In 1977 *S*. Newport was responsible for the largest outbreak of salmonellosis attributed to beef that has been reported to the CDC, affecting 200 individuals; the outbreak was traced and epidemiologically linked to consumption of a common source of roast beef. *S*. Newport and *S*. Typhimurium are currently the only multidrug-resistant serovars identified in outbreaks attributed to beef (Laufer et al., 2015). Over the past three decades, *S*. Newport acquired multiple drug resistance. *Salmonella* Newport-MDRAmpC isolates are resistant to ampicillin, amoxicillin/clavulanic acid, cephalothin, cefoxitin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, and ceftiofur, and exhibit decreased susceptibility to ceftriaxone, which has complicated treatment and necessitated the search for novel approaches to pathogen reduction (Gupta et al., 2003; Laufer et al., 2015; Varma et al., 2006). One strategy currently
being evaluated seeks to prevent or significantly reduce enteric colonization of domesticated livestock with pathogenic bacteria using phage treatment (Sulakvelidze, 2013).

The reduction of foodborne pathogens requires a comprehensive intervention program encompassing pre-harvest (at the farm and during transport/shipment to the processing plants) and post-harvest portions. One proposed pre-harvest intervention to reduce sources of foodborne pathogen contamination is the use of specific phages to selectively reduce or eliminate susceptible bacteria from selected environments (Jassim and Limoges, 2014). Phage treatment can address food safety concerns by not contributing to antibiotic residues in animal meat, meat products, eggs, and milk, during treatment of specific bacterial infections in animals (Tiwari et al., 2014). It has already been demonstrated that when infected animals are administered phage treatments, there is little danger of susceptible animals at the same the premises (environmental exposure) or with which they were in contact (direct contact exposure) becoming infected (Smith and Huggins, 1983). Bactericidal phages may thus provide a feasible natural and nontoxic approach to controlling human pathogens in livestock (Jassim and Limoges, 2014).

A five bacteriophage cocktail (Appendix 2.1) to reduce S. Newport in calves was evaluated by Hyland et al. Three of the five S. Newport-targeted phages were shown to morphologically resemble members of the Myoviridae (S41 and S50) bacteriophage family, two resemble the Siphoviridae (S40 and S44), and one resembles the Podoviridae (S11). Hyland et al found that oral phage cocktail treatment decreased fecal shedding of S. Newport, and -- when administered at the onset of clinical signs-- decreased the severity of clinical illness in calves. Calves treated with the phage cocktail had significantly reduced rectal temperatures (e.g., normal or only slightly elevated) and significantly less fecal bacterial shedding than did untreated calves (Hyland et al., unpublished).

One challenge to phage treatment is the rapid development of phage resistance (Capparelli et al., 2010a; León and Bastías, 2015; Mahony et al., 2011; Sulakvelidze, 2013). Phage resistance of E. coli in phage-treated calves has been studied, but little work has been performed to determine the effect phage resistance in Salmonella has on the disease (Smith and Huggins, 1983). The fitness cost of phage-resistant bacterial strains is particularly high and may cost the bacterium in virulence reduction. Also, due to the chance of phage-resistance conferring increased virulence and the potential for transfer of phage-encoded virulence factors into recipient bacteria in vivo, studies on phage-resistant mutants should be performed for any phage
that is under consideration for commercial application (León and Bastías, 2015; Mahony et al., 2011). Therefore, in the current study, we hypothesized that phage-resistant mutants from the Hyland et al. model of S. Newport-infected, phage treated calves would not be able to colonize cattle, and therefore would not pose a threat to uninfected animals.

2.3. Materials and Methods

2.3.1. Bacteria and Bacteriophage Isolates

S. Newport strain 3596 was originally isolated from a diseased cow by the Auburn University College of Veterinary Medicine Diagnostic Bacteriology Laboratory. All other strains used in all experiments was derived from this isolate. A spontaneous nalidixic acid resistant (NalR) mutant and rifampin resistant mutant (RifR) from S. Newport 3596 was selected to serve as antibiotic resistant marked strains (Miller, 1972). The phage-resistant S. Newport (S. Newport MutΦ) described later was generated from the nalidixic acid-resistant marked strain of S. Newport (NalR).

Five bacteriophage isolates (S11, S40, S41, S44, S50) originally isolated from Salmonella-containing diagnostic cultures previously characterized by Hyland et al were used in this study (Hyland et al., unpublished). These five phages showed lytic activity against S. Newport in vitro and therapeutic effects in vivo in a S. Newport infection calf model (Hyland et al., unpublished).

To amplify phage to produce high titer stocks, 50mL of log phase S. Newport cells growing in Luria-Bertani broth (LB; Difco LB Broth, Miller, Becton, Dickinson, and Company, Sparks, MD) containing 1mM magnesium (LBM) was inoculated with 0.5mL of the purified phage solution. The lysate was incubated overnight at 37 degrees Celsius (°C), and then was pelleted at 12,500 times gravity (x g) for 15 minutes. The resulting supernatant was filter sterilized through a 0.2µm filter (Sterile Syringe Filter with 0.2µm Polyethersulfone Membrane, VWR International). To enumerate the phage in each supernatant, a double agar overlay method was used for titration. S. Newport cells were cultured to log phase, then diluted to an absorbance, or optical density, measured at a wavelength of 620 nm (OD$_{620}$) of 0.8 to 1.0. Serial dilution of each phage solution were performed, and 0.2mL of the S. Newport cells was mixed with 10µL of the phage solution. The cells were incubated with the phage for ten minutes before adding 3mL (LBM with 0.7% BD Bacto agar and 1mM tetrazolium dye) overlay or top agar and pouring the mixture onto an underlay or bottom agar that is Luria-Bertani broth (Difco LB Broth, Miller)
containing 1mM magnesium and 1.5% agar (LBM agar). The phage plaques were enumerated to obtain the plaque forming units per mL (PFU/mL) (Kropinski et al., 2009). To determine lytic activity of phage on a host strain, a double agar overlay was used with host bacterium but no phage added to the top agar. After the overlay agar was allowed to harden for 30 minutes, a 10µL drop of phage supernatant was placed on the top agar. The phage drop was allowed to dry; the plates were inverted and incubated overnight at 37°C. Lytic activity on the host strain was determined by examining the plates for a clearing of bacterial growth at the location where the phage supernatant drop was placed (Kutter, 2009).

2.3.2. Experimental Calves, Pre-inoculation Culturing, and Care

Calves were obtained from the Auburn University College of Veterinary Medicine herd and acclimated in a Biosafety Level 2 animal facility designed for the purpose of working with infectious diseases in livestock. A total of 7 calves was utilized (3 for bacteriophage challenge experiments and 6 for S. Newport MutΦ experiments; calf P1 and P2 was also calf #3 and #4) throughout the entirety of this report. Temperature, fecal score, and clinical signs were reported twice daily and a fecal sample was obtained daily for Salmonella phage isolation. Fecal scores (1-5) were defined as: 1 = dry, 2 = normal, 3 = soft, 4 = diarrhea and 5 = watery or bloody diarrhea. Fever in calves was set as rectal body temperature above 103.0 degrees Fahrenheit (°F) (Radostits et al., 2000). Pre-inoculation fecal samples were screened for the presence (+ or -) of Salmonella species by inoculating a sample swab into 5mL of Tetrathionate broth tubes (TTh; Difco Tetrathionate Broth Base, BD), incubating overnight at 37°C, and streaking the resultant inoculum on Xylose Lysine Agar (Difco XLT4 Agar Base, BD) supplemented with Tergitol 4 (XLT4; Difco XLT4 Agar Supplement, BD). Pre-inoculation TTh broths were filtered by a 0.45µm syringe filter (Sterile Syringe Filter with 0.2µm Polyethersulfone Membrane, VWR International) and supernatants were screened for presence of lytic activity against S. Newport by the phage spot test described earlier to confirm the absence of S. Newport-targeted phage. At the conclusion of each portion of the calf experiments, the calf or calves were humanely euthanized and disposed by incineration in order to ensure Salmonella was not transmitted beyond the animal isolation facility. The protocols for handling Salmonella in the laboratory and in the animal facilities were approved by the Auburn University Institutional Biosafety Committee. In addition, all animal procedures followed protocols described in the Guide for the Care and Use...
of Laboratory Animals: Eighth Edition, and were approved by the Auburn University Institutional Animal Care and Use Committee.

2.3.3. **Bacteriophage Calf Challenge and Enumeration from Calf Feces**

To determine if orally administered phages invoked disease signs, two calves (n=2) were administered two doses of $1 \times 10^{11}$ plaque forming units (PFU’s) of the *Salmonella* phages S11, S40, S41, and S50 orally using a dosage syringe on day 0 and day 1. Temperature, fecal score, and clinical signs were reported twice daily and a fecal sample was obtained daily for *Salmonella* phage isolation. Fecal samples were screened for the presence (+ or -) of *Salmonella* species by inoculating a sample swab into 5mL TTH broth, incubating overnight at 37°C, and streaking the resultant inoculum onto XLT4 agar. *Salmonella* phage PFU’s within the fecal samples were enumerated by first making a 10-fold dilution of feces in salts-magnesium (SM) buffer. This 10-fold dilution was centrifuged to pellet fecal material at 12,000 x g for 15 minutes, and the supernatant was filtered through a 0.45µm syringe filter. For detection of phage plaques, *S.* Newport cells were cultured to log phase, then diluted to an OD$_{620}$ of 0.8 to 1.0. Serial 10-fold dilutions of each sample were performed, and 0.2mL of the *S.* Newport cells was mixed with 100µL of the fecal supernatant. The cells were incubated with the supernatant for twenty minutes before adding 3mL top agar with 1mM tetrazolium dye (Kropinski et al., 2009) and pouring the mixture onto an LBM bottom plate. The plates were allowed to solidify, then incubated overnight at 37°C. The phage plaques were enumerated to obtain the (PFU/mL) of the 10-fold dilution and this was multiplied by 10 to give plaque forming units per gram of feces (PFU/g). At the conclusion of each experiment, the calves were humanely euthanized.

In a second experiment, following acclimation, a calf (n=1) was orally inoculated with a phage at a dose of $1 \times 10^{10}$ PFU’s with phage S11. Additional phages were administered orally every three days in the order of phage S40, S41, S50, and finally S44. Fecal samples were collected daily, fecal scores made twice daily, and body temperature measured twice daily. Fecal samples collected daily were sub-cultured in TTh as described earlier for *Salmonella* presence during the experiment. One gram from fecal samples collected at 12, 24, 48, and 72 hours post phage inoculation and was phage enumerated as previously described with the exception of double agar plates were incubated 8-18 hours at 37°C for phage S11, S40, S44, S50 and at 26°C for phage S41.
2.3.4. Generation of a Bacteriophage-Resistant S. Newport Mutant

The method used to generate a phage-resistant mutant of *Staphylococcus aureus* by Rosanna Capparelli et al. (2010b) was used to generate a phage-resistant *S. Newport* isolate (Capparelli et al., 2010b). A S50 phage-resistant *S. Newport* strain (*S. Newport MutΦ*) was isolated by plating dilutions of an overnight culture of the phage susceptible spontaneous NalR mutant of an *S. Newport* (*S. Newport NalR*) strain onto LBM agar containing increasing concentrations of phage S50. Colonies growing on the highest phage concentration were selected and sub-cultured twice on LBM agar in the absence of phage. Colonies were challenged with all five *S. Newport* cocktail phage by spot lysis. Isolates showing the least lysis from the five *S. Newport* phage cocktail were sub-cultured twenty times in the absence of phage and re-screened for phage resistance to ensure the stability of the resistance.

To determine if there were any differences in growth in LBM broth, *S. Newport* 3596 (wild-type), *S. Newport NalR*, *S. Newport RifR*, and *S. Newport MutΦ* were grown in four different flasks containing 12.5mL of LBM broth; each was inoculated with an overnight culture of *S. Newport* 3596, *S. Newport NalR*, *S. Newport RifR*, or *S. Newport MutΦ* cells to a final OD_{620} of 0.5. Each flask was incubated at 37°C with aeration and OD_{620} readings taken at time 0, and then at approximately 30 minute intervals for 6 hours.

To determine lytic activity of the phage on the phage-resistant mutant, flasks containing 12.5mL of LBM were inoculated with an overnight culture of *S. Newport* cells to a final OD_{620} of 0.5. One of five phages (S11, S40, S41, S44, and S50) was added to a flask with a multiplicity of infection (MOI) equal to 1.0. A flask containing only *S. Newport* with 100µL SM buffer was used as a control. Each flask was incubated at 37°C with aeration and OD_{620} readings taken at time 0, and then at approximately 30 minute intervals for 6.5 hours. These lysis curves at an MOI equal to 1.0 were performed on the parent *S. Newport NalR* and *S. Newport S. Newport MutΦ* to determine phage lytic activity resistance in broth. Adsorption kinetics for S50 on both parent *S. Newport* and mutant *S. Newport MutΦ* was performed to determine if the mutation responsible for phage resistance was affected a potential surface molecule that conferred resistance to phage S50. A mid-log phase culture of *Salmonella* was diluted to give 10mL with an OD_{650} of 0.1–0.2. From the cell suspension, 9mL was added to a 125mL flask labeled “A” and 9 ml of LBM broth added to a 125mL flask labeled “C”. At time = 0, 1mL of warmed S50 phage suspension with a titer of 1 – 3x10^5 (PFU/mL) to flask A and 1ml of S50 phage to flask C. At one-minute
intervals for ten minutes, 0.05mL aliquots from flask A was added to chilled tubes containing three drops of chloroform. Tubes were vigorously vortexed for 10 seconds before being placed on ice. After ten minutes, 0.05mL samples from flask C were added to tubes C1 and C2 that contained chloroform. From each sample tube, 0.1mL was inoculated into the molten top agar and poured onto a bottom agar plate using the double agar overlay method as described earlier. The Salmonella cell suspension was diluted to the $10^{-6}$ dilution and 0.1mL aliquots from the $10^{-4}$ to $10^{-6}$ dilutions was spread onto LB agar plates to determine (CFU/mL) of the cell suspension. The phage plaque counts from each aliquot from 0 to 10 minutes was plotted using a semi-log scale and a line of best fit was determined. Time = 0 was the average number of plaques on plates from C1 and C2. The adsorption rate constant ($k$) was calculated from the formula: $k = \frac{2.3}{Bt} \log \frac{P_0}{P}$ where $k$ is the adsorption rate constant, in mL/minute; $B$ is the concentration of bacterial cells; $t$ is the time interval in which the titer falls from $P_0$ (original) to $P$ (final) (Kropinski, 2009). However, if no difference in absorption kinetics is found, this would suggest that there may be some internal mechanism of resistance.

2.3.5. Calf Challenge with the Bacteriophage-Resistant S. Newport Mutant

Following acclimation and pre-inoculation screenings, a pair of calves (n=2) was inoculated orally with the phage resistant mutant at a dose of $7.45 \times 10^9$ CFU’s. Fecal samples were collected daily, feces were scored twice daily, and body temperature measured twice daily. Fecal samples were screened for the presence or absence (+ or -) of S. Newport MutΦ by inoculating a sample swab into 5mL TTH broth containing Nal at a concentration of 35 µg/mL, incubating overnight at 37°C, and streaking the resultant inoculum on XLT4 + Nal plates. S. Newport MutΦ CFU’s within the fecal samples were enumerated by making 10-fold dilutions of feces in Dulbecco’s phosphate buffered saline without calcium and magnesium (PD) and spread-plating 100µL of each dilution onto XLT4 + Nal plates in duplicate. All plates were incubated at 37°C and colonies were counted after 24-48 h of incubation. The feces also were screened for phages by the phage spot test described previously. At the conclusion of each experiment, the calves were humanely euthanized.

Following acclimation and pre-inoculation screenings, a second pair of calves (n=2) was challenged with a higher dose ($1.47 \times 10^{10}$ CFU’s) of the phage resistant mutant to determine if the mutant could cause disease at a high challenge dose. Fecal samples were collected daily and processed as previously described, feces were scored twice daily, and body temperature
measured twice daily. At the conclusion of the experiment, both calves were humanely euthanized.

2.3.6. Competition Experiment in Calves Between Parent and Bacteriophage-Resistant \textit{S. Newport}

Two calves (n=2) were obtained from the Auburn University College of Veterinary Medicine herd. Following acclimation and pre-inoculation screenings as described previously, both calves were inoculated orally with 8.5x10^9 CFU’s of the parent \textit{S. Newport} strain (\textit{S. Newport} Rif^R) and 1.11x10^{10} CFU’s of the phage resistant mutant \textit{S. Newport} Mut^Φ, giving a total \textit{S. Newport} inoculation dose of 1.96x10^{10} CFU’s with a ratio of 1:1.3 parent:mutant. Nal (35ug/ml) or rifampicin (20ug/ml) was added to the XLT4 medium to select for the mutant or parent strain, respectively. Fecal samples were collected and cultured daily, fecal scores determined twice daily, and body temperature measured twice daily.

Fecal samples were screened for the presence (+ or -) of \textit{S. Newport} Rif^R and/or \textit{S. Newport} Mut^Φ by inoculating a sample swab into 5mL TTH broth, incubating overnight at 37°C, and streaking the resultant inoculum on XLT4 + Nal or XLT4 + Rif plates. \textit{S. Newport} Rif^R and \textit{S. Newport} Mut^Φ CFU’s within the fecal samples were enumerated by making 10-fold dilutions of feces in PD and spread-plating 100µL of each dilution onto XLT4 + Nal or XLT4 + Rif plates in duplicate. The feces were screened for phages by phage spot test described previously. All plates were incubated at 37°C and colonies were counted after 24-48 h of incubation. On the final day of the experiment, 14 colonies of the \textit{S. Newport} Mut^Φ were isolated. These 14 colonies of \textit{S. Newport} Mut^Φ were phage spot tested as described earlier with phages S11, S40, S41, S44, and S50 to determine if the phage resistant phenotype was maintained following transit through the calves. At the conclusion of each experiment, the calves were humanely euthanized.

2.4. Results

2.4.1. Bacteriophage Calf Challenge and Enumeration from Calf Feces

In a control experiment, two calves were inoculated with a high dose of the phage cocktail to test for toxic effects. Calves P1 and P2 were orally administered 10^{11} PFU’s of each phage S11, S40, S41, and S50 on day 0 and day 1. Fecal phage counts were enumerated daily (Fig. 2.1). Calf P1 initially shed the total phage at a concentration of Log_{10} = 4.5 PFU/gram of feces. The fecal shedding of phage decreased until day 6 when the phage could no longer be enumerated in feces. Calf P2 initially shed the total phage at a concentration of Log_{10} = 3.95
PFU/gram of feces. The fecal shedding of phage increased on day 2, then decreased until day 5 when the phage could no longer be enumerated in feces. The individual phage could not be easily distinguished from one another on the plaque plates from either calf.

A third calf (P3) was orally inoculated on day 0 with a phage S11 at a dose of 1x10^{10} plaque forming units (PFU’s). No phage was enumerated from feces 12, 24, or 48 h post inoculation (Fig. 2.2). On day three, 1x10^{10} PFU’s of phage S40 was administered to the calf. Twelve hours after inoculation, Log_{10} = 3.15 PFU/gram was recovered from feces. Twenty-four hours post S40 inoculation, Log_{10} = 2.40 PFU/gram phage was recovered from feces. Forty-eight hours post S40 inoculation, no phage was recovered from feces. On day six, 1x10^{10} PFU’s of phage S41 was administered to the calf. Twelve hours after inoculation, Log_{10}=2.0 PFU/gram phage was recovered from feces. Twenty-four hours post S41 inoculation, phage was not recovered from feces. On day nine, 1x10^{10} PFU’s of phage S50 was administered to the calf. Twelve hours after inoculation, Log_{10}=2.0 PFU/gram phage was recovered from feces. Twenty-four hours post S50 inoculation, Log_{10}=2.85 PFU/gram phage was recovered. Forty-eight hours post S50 inoculation, Log_{10}=2.30 PFU/gram phage was recovered. Seventy-two hours post S50 inoculation, phage was not recovered from feces. On day twelve, the calf was administered a dose of 1x10^{10} PFU’s with phage S44. Twelve hours after inoculation, no phage was enumerated from feces, and the calf was humanely euthanized on day 15. The calf was culture negative for Salmonella spp by tetrathionate enrichment broth throughout the experiment. No clinical disease observed in this calf, as it had a fecal score of 2 (Normal) and maintained a normal body temperature for the duration of the experiment.

2.4.2. **Generation of a Bacteriophage Resistant S. Newport Mutant**

Multiple phage-resistant mutants of the S. Newport strain were isolated by plating dilutions of an overnight susceptible culture of the S. Newport NalR strain onto LBM agar containing increasing concentrations of phage S50. Colonies expressing the S50 resistance phenotype by spot test were sub-cultured 20 times and re-tested for phage resistance to S50 to ensure that the phage resistant phenotype was maintained. Individual isolates were then challenged by spot tests to each of the other cocktail phage in an effort to find a mutant resistant to all 5 cocktail phage. Some of the S50 resistant isolates were susceptible to all the other phages (S11, S40, S41, S50) The phage phenotypes observed ranged from mutants with only resistance to S50 up to mutants resistant to four out of five of the cocktail phages. Because no isolate
showed resistance to all 5 cocktail phages, the isolate that showed resistance to S11, S40, S41, and S50 by spot lysis was chosen for further characterization and studies.

Because the phage resistant mutant was destined for virulence testing in calves, we first determined if the mutation(s) that conferred phage resistance affected growth in vitro. No difference in growth in LBM broth between the S. Newport 3596 (wild-type), S. Newport NalR, S. Newport RifR, and S. Newport MutΦ. Only the data for the parent (S. Newport NalR) and the phage-mutant (S. Newport MutΦ) is shown in Figure 2.3. There were no differences in growth from all strains. To confirm the phage resistance phenotype indicated by the spot lysis tests, the parent and mutant strains were grown in LBM broth and challenged individually with each cocktail phage in early exponential growth. The parent strain showed a degree of lytic activity by each of the phage as shown in Figure 2.4. The S. Newport MutΦ challenged by the different phages showed a much different lysis pattern (Fig. 2.5) compared to the parent (Fig. 2.4). Only phage S44 showed lytic activity against S. Newport MutΦ. There is an apparent decrease of in the optical density for S44, but still it is not as pronounced as the decrease seen in the parent strain. The adsorption kinetics of S50 for both parent S. Newport and mutant S. Newport MutΦ was performed to determine if the mutation responsible for phage resistance affected a surface molecule that conferred resistance to phage S50. The rate of attachment of S50 phage to the parent was $2.69 \times 10^{-9}$ mL/min. The adsorption rate constant for S50 on S. Newport MutΦ was 0.00 mL/min. This result shows that phage S50 did not attach to S. Newport MutΦ, indicating that a surface receptor has been modified or is no longer expressed, preventing S50 binding.

2.4.3. Calf Challenge with the Bacteriophage Resistant S. Newport Mutant

After a 16 day acclimation period with no positive pre-inoculation cultures of Salmonella or phage activity, calf #1 (8 weeks old) and calf #2 (10 weeks old) was orally challenged with phage resistant mutant S. Newport MutΦ at a dose of $7.45 \times 10^9$ CFU’s on day zero (Fig. 2.6). Both calves showed measurable shedding of S. Newport MutΦ approximately log10 CFU of 3.0 of S. Newport MutΦ per gram of feces 24 hours post inoculation. By 48 hours post inoculation, calf #2 had no measurable shedding of S. Newport MutΦ and calf #1 was shedding of S. Newport MutΦ log10 CFU of 2.75 of S. Newport MutΦ. By day 3 post inoculation, both calves was not shedding measurable numbers of S. Newport MutΦ. By day 4 post inoculation, S. Newport MutΦ was not detectable by enrichment broth in either calf. Both calves maintained normal body
temperatures and exhibited no signs of diarrhea throughout the duration of 25 days of the experiment.

After a 10-day acclimation period with no positive pre-inoculation cultures of *Salmonella* or phage activity, calf #3 (6 weeks old) and calf #4 (6 weeks old) was orally challenged with phage resistant mutant *S. Newport MutΦ* at a dose of $1.47 \times 10^{10}$ CFU’s on day zero (Fig. 2.7). Both calves showed measurable shedding of *S. Newport MutΦ* approximately $\log_{10}$ CFU of 3.1 (calf #3) and 3.7 (calf #4) of *S. Newport MutΦ* per gram of feces 24 hours post inoculation. By 48 hours post inoculation, both calves were not shedding measurable numbers of *S. Newport MutΦ*. On day 8 post inoculation, calf #4 showed measurable shedding of *S. Newport MutΦ* approximately $\log_{10}$ CFU of 2.3 of *S. Newport MutΦ* per gram of feces. *S. Newport MutΦ* positive by enrichment broth seized on day 8 for calf #3 and on day 10 for calf #4. Calf #3 had a fecal score of 4 (Diarrhea) on day 2 through day 4 post inoculation, while calf #4 had a fecal score of 4 (Diarrhea) on day 4 only. Calves exhibited a fecal score of 3 (Soft stool) prior to days of diarrhea followed by a fecal score of 2 (Normal) after diarrhea seized. Both calves maintained normal body temperatures throughout the duration of 16 days of the experiment.

### 2.4.4. Competition Experiment in Calves Between Parent and Phage-Resistant *S. Newport*

Following an acclimation of 6 days, two 6 to 8 week old calves (#5 and #6) was orally administered on day zero with an inoculum size of $8.5 \times 10^9$ CFU’s of the parent *S. Newport strain* (*S. Newport RifR*) and $1.11 \times 10^{10}$ CFU’s of the phage resistant mutant *S. Newport MutΦ*, giving at total *S. Newport inoculation dose of 1.96$ \times 10^{10}$ CFU’s with a ratio of 1:1.3 parent:mutant. In the calf #5, maximum $\log_{10}$ CFU of *S. Newport MutΦ* per gram of feces ranged from 5.04 to 6.11 between days 9 and 18, then consistently decreased until bacterial numbers were a $\log_{10}$ CFU of *S. Newport MutΦ* per gram of feces of 2.74 when the experiment was terminated (Fig. 2.8). In the calf #6, maximum $\log_{10}$ CFU of *S. Newport MutΦ* per gram of feces ranged from 5.08 to 6.04 between days 12 and 21, then consistently decreased until bacterial numbers were a $\log_{10}$ CFU of *S. Newport MutΦ* per gram of feces of 2.88 when the experiment was terminated (Fig. 2.6).

In the calf #5, elevated $\log_{10}$ CFU of *S. Newport RifR* per gram of feces ranged from 4.13 to 4.40 between days 7 and 10, then consistently decreased until *S. Newport RifR* bacterial numbers were lower than the detection limit by day 21 post inoculation. In the calf #6, elevated $\log_{10}$ CFU of *S. Newport RifR* per gram of feces ranged from 3.94 to 4.75 between days 12 and
16, then consistently decreased until S. Newport Rif\textsuperscript{R} bacterial numbers were lower than the detection limit by day 24 post inoculation.

The maximum temperature of calf #5 ranged from 103.2\textdegree F to 104.9\textdegree F between days 3 and 5, then returned to normal (less than 102.5\textdegree F). The maximum temperature of calf #6 ranged from 103.1\textdegree F to 104.9\textdegree F between days 2 and 4, then returned to normal (less than 102.5\textdegree F). Both calves had a fecal score of 5 (Watery/Bloody Diarrhea) beginning 24 hours post inoculation and ending on day 6 for calf #5 and day 4 for calf #6. This fecal score fluctuated from a fecal score of 3 (Soft Stool) to a fecal score of 4 (Diarrhea) for the rest of the duration of the experiment. Calf #5 appeared to be lethargic on day 2 through day 4 post inoculation. Calves #5 and #6 were positive by enrichment broth for both parent and phage-resistant mutant throughout the entirety of 24 day of the experiment. S. Newport Mut\textsuperscript{Φ} colonies isolated from feces from day 24 post inoculation maintained phage-resistance phenotype displayed prior to inoculation with only S44 exhibiting lytic activity against the mutant.

2.5. Discussion

The acquisition of multiple drug resistance by S. Newport (and other \textit{Salmonella} serovars) has complicated treatment and necessitated the search for novel approaches to pathogen reduction, as has the impending removal of antibiotic use for growth promotion. The strategy being evaluated by our research group is phage treatment for the reduction of \textit{S.} Newport in dairy calves.

We showed that a phage cocktail challenge at the high dose of $10^{11}$ PFU’s each did not cause any clinical disease in these calves. Each calf had a fecal score of 2 (Normal) for the duration of the experiment. Both calves maintained a normal body temperature during the duration of the experiment, and both calves were culture negative for \textit{Salmonella} species by tetrathionate enrichment broth throughout the experiment. Although the individual phage could not be easily distinguished from one another on the plaque plates, it was obvious that one or more isolate was shed for several days during and after inoculation. We also showed that phage can be recovered from feces twelve hours after oral ingestion even without inoculation with a bacterial host.

Additionally, the current study focused on another area of concern with phage treatment in the development of phage resistance and the concern of how this phage resistance might alter the virulence of the bacterium. Our S50 bacteriophage-resistant mutant of \textit{S.} Newport (S.
Newport Mut$^\Phi$ does not appear to be any different in colony shape, morphology, or growth in broth when compared to the parent strain in the absence of phage. This is in contrast to other researchers who have looked at phage-resistance and the effect it has on the growth of the bacterial organism, such as in *Staphylococcus aureus* which phage-resistance caused decreased growth rate, reduced expression of several virulence factors, and production of a capsular polysaccharide (Capparelli et al., 2010b). Another example of decreased fitness from phage-resistance was found in *Serratia marcescens*, in which phage-resistance decreased the maximum population size and decreased motility following growth at higher temperatures (37°C) (Friman et al., 2011). Other studies on *Escherichia coli*, found that phage-resistant mutants had a lower growth rate in broth and smaller colonies on agar, than the parent strain (Demerec and Fano, 1945).

Phage-resistance is typically a spontaneous mutation and the resistance is not induced by the application of the phage, but occurs in the bacterium before the phage comes into contact with the bacterium (Beale, 1948). We found in our experiment that using one phage for selective pressure to select for a spontaneous mutation that confers phage-resistance in *S. Newport* also confers resistance to four out of five of our cocktail phage. This result is similar to an investigative study of *Escherichia coli* that showed exposure to one of seven phage (T1 to T7) yielded many phage-resistant mutants that had resistance to more than one phage that the authors believe arose from one-step mutations (Demerec and Fano, 1945). The parent and phage-resistant mutant *S. Newport* strains do differ in their susceptibility to lysis by the cocktail phages, however, with the mutant showing complete resistance to S11, S40, S41, and S50, and decreased susceptibility to phage S44. The mutant phenotype is surprising because it was selected following exposure to just one phage, S50. Interestingly, the phage-resistant mutant was still susceptible to phage S44. This sensitivity would suggest that S44 most likely utilizes a separate receptor from the other four phages. At this time, we can speculate that S11, S40, S41, and S50 might all use the same surface molecule as their respective phage receptors. If the other four phages do not use the same receptors to identify the bacterium, this would mean that the mutation that conferred phage-resistance could be in an underlying system responsible for the phenotypic production of each of the four phages’ receptors.

The phage-resistant mutant was administered orally at a dose of 7.45x10$^9$ CFU’s to two calves to determine if it could colonize, cause disease, and be shed in the feces. Fecal shedding
of $\geq 10^2$ CFU/g of the mutant ceased in both calves by post-inoculation day three (Fig. 2.6), which is a shorter shedding time compared to the parent strain in calves (Appendix 2.2; Hyland et al., unpublished). Neither calf inoculated with the phage-resistant mutant showed any disease signs, while calves challenged with the parent strain at this dose show typical signs salmonellosis (fever and diarrhea). To determine if the mutant’s apparent attenuation was dose-dependent, a second pair of calves was inoculated with $10^{10}$ CFU’s of the mutant. Again, fecal shedding of $\geq 10^2$ CFU/g of the mutant ceased by day two in both calves post-inoculation, although Calf 4 shed just countable levels on day 8 (Fig. 2.7). Both calves inoculated with the higher dose of the phage-resistant mutant S. Newport strain showed abbreviated signs of diarrhea and neither showed any signs of fever. The calves which received the higher inoculum of the phage resistant mutant shed detectable levels of the salmonellae longer in feces and had diarrhea compared to those that received the lower dose (Fig. 2.6 vs. Fig. 2.7). However, this reduced fecal shedding and diarrhea indicates that even a high dose of the mutant caused is not what is typically seen in a case of salmonellosis. This was a much milder form of salmonellosis, suggesting the mutation(s) conferring phage resistance also attenuated virulence. This finding is not unlike those reported in other studies, where the mutation responsible for the phage resistance attenuated the bacterium (Capparelli et al., 2010b; Laanto et al., 2012; Scott et al., 2007; Shamim Hasan Zahid et al., 2008; Smith and Huggins, 1983).

A third pair of calves was challenged with a total dose of $1.96 \times 10^{10}$ CFU’s composed of a 1:1.3 ratio of parent:mutant in a competition experiment designed to determine how well the mutant competed against the parent strain in vivo. Both strains showed an unusual shedding pattern in this experiment. Hyland et al showed that control calves shed S. Newport at countable numbers through day 12 when administered a dose of around $5 \times 10^9$ CFU’s of the parent strain (Appendix 2.2; Hyland et al., unpublished). Our competition experiment showed countable numbers of the S. Newport Mutant shed out to day 24, when the experiment was terminated (Fig. 2.8 and Fig. 2.9), compared to its much reduced shedding time when administered without the parent strain (Fig. 2.6 and Fig. 2.7).

The calves involved in the competition experiment had fecal shedding of high numbers of both phage-resistant mutant and parent S. Newport, with clinical illness with fever from day 3 to day 6 for one calf and from day 2 to day 4 for the second calf. Despite continued high shedding, disease signs were mild to absent after day 6. The very high inoculum of parent and mutant
strains combined could be responsible for this high shedding result. Oral inoculations of *Salmonella* spp at doses between $10^8$ and $10^{11}$ CFU’s can result in lethal infections (Costa et al., 2012; Santos et al., 2001). A study of older calves (75 days of age) inoculated with *S*. Newport found that doses of $10^9$ and $10^{10}$ CFU’s required euthanasia 48-72 hours and 36 hours post-infection, respectively. And Snider et al. (2014) found that *S*. Newport doses had to be adjusted 10-fold lower than doses of *S*. Typhimurium, due to the higher virulence of *S*. Newport.

The conflicting calf fecal shedding results with the *S*. Newport Mut$^\Phi$ strain are intriguing. When inoculated singly, the mutant caused no disease in the calves and was not able to survive to be shed in the feces, which would suggest that the phage-resistance mutation(s) attenuated virulence. In contrast, in the competition experiment where both parent and phage-resistant mutant of *S*. Newport were administered together, shedding of the *S*. Newport Mut$^\Phi$ was prolonged. It appears that the presence of the parent strain enabled the mutant to survive and be excreted fecally.

The explanation for these conflicting results most likely involves the mutation(s) that conferred phage-resistance on *S*. Newport Mut$^\Phi$, which we speculate also disabled the ability of the mutant to invade the epithelium in the gastrointestinal tract of the calves. *Salmonella* species are able to induce intestinal inflammation by virulence factors (encoded on *Salmonella* Pathogenicity Islands 1 and 2) (LaRock et al., 2015) that results in the production of large amounts of nitric oxide radicals and reactive oxygen species in the lumen of the gut. Thiosulfate ($S_2O_3^{2-}$) can be oxidized to tetrathionate ($S_4O_6^{2-}$), which *Salmonella* species, unlike other coliforms, can utilize as terminal electron acceptor in the anaerobic environment of the intestinal lumen because they produce tetrathionate reductase, whose gene is encoded on SPI-2 (Rohmer et al., 2011). *Salmonella*-mediated inflammation generates tetrathionate in the intestine (Winter et al., 2010). This provides *Salmonella* species with a growth advantage in an inflamed gut (Thiennimitr et al., 2011). A mutant unable to invade gut epithelium would not induce inflammation and thus no tetrathionate would be produced, resulting in no growth advantage and clearance of the mutant. However, when inoculated with the parent invasive strain, the inflammatory response caused by invasion of the parent would produce enough tetrathionate to maintain the mutant in the gut lumen, and be shed in the feces, while the parent would invade and cause signs of disease. This would explain the prolonged fecal shedding of *Salmonella* in the competition experiment.
We speculate that the mutation responsible for the phage resistance and invasion-minus phenotype of the *S. Newport Mut* strain might interfere with one of the type three secretion systems (T3SSs) that are important for pathogenesis of *Salmonella* spp. A model for what we hypothesize that is occurring in the lumen of the calves can be seen in Figure 2.10. The type three secretion system one (T3SS-1) located on SPI-1 is important for invasion of epithelial cells and the type three secretion system two (T3SS-2) located on SPI-2 is important survival in macrophages (Lahiri et al., 2010). SPI-1-mediated colonization of intestinal tissues appears to be essential for bovine enteritis, but SPI-2 mutants are only mildly attenuated in calves: an SPI-2 (*spiB*) mutant of *S. Typhimurium* has been shown to cause lethal morbidity and diarrhea comparable to a wild type *S. Typhimurium* in calves infected with $10^{10}$ CFU’s (Tsolis et al., 1999). Due to knowledge that SPI-2 mutants are only mildly attenuated in calves, but our *S. Newport Mut* appears profoundly attenuated in a similar model, we suggest that the mutation in our phage resistant *S. Newport* mutant must interfere with the SPI-1- genes encoding the T3SS-1 injectisome. Full genome sequencing could confirm our suspicion by identifying the location of the mutation.

2.6. Conclusions

The purpose of this study was to examine concerns regarding bacteriophage therapy. It appears that bacteriophage by itself did not cause any significant disease and that phage can be found in the feces as early as twelve hours after being orally ingested. The generation of a multi-bacteriophage-resistant *S. Newport* mutant by exposure to only one of these bacteriophages indicates that four of our five cocktail bacteriophages may recognize the same receptor on their *Salmonella* host. The conflicting calf fecal shedding results with the bacteriophage-resistant mutant suggests that the mutation(s) may have reduced its ability to colonize its host. The location(s) of the mutation(s) is currently unknown, but we believe that it somehow interferes with the formation of the T3SS-1. Without the T3SS-1, inflammation is not induced and the phage-resistant *S. Newport* is not able to out-compete the other microbiota.

2.7. References


2.8. Chapter 2 Tables and Figures

Figure 2.1. Fecal bacteriophage counts shed from two calves and rectal temperatures following challenge of 1x10^{11} plaque forming units (PFU’s) of the Salmonella bacteriophages S11, S40, S41, and S50 orally on day 0 and day 1.
Figure 2.2. Fecal bacteriophage counts shed from one calf following challenge of $1 \times 10^{10}$ plaque forming units (PFU’s) with phage S11 on day 0, $1 \times 10^{10}$ PFU’s of phage S40 on day 3, $1 \times 10^{10}$ PFU’s of phage S41 on day 6, $1 \times 10^{10}$ PFU’s of phage S50 on day 9, and finally $1 \times 10^{10}$ PFU’s of phage S44 on day 12.
Figure 2.3. Optical density (OD$_{620}$) measurements of exponential and early stationary growth of *Salmonella* Newport parent strain and *Salmonella* Newport bacteriophage-resistant mutant strain in LBM broth. shaking at 37°C.
**Figure 2.4.** Optical density (OD$_{620}$) measurements of growth of *S*. Newport parent strain in LBM broth cultures challenged with bacteriophages (MOI=1.0) at time 0. Control flask contained cells in LBM buffer without bacteriophage.
Figure 2.5. Optical density (OD$_{620}$) measurements of growth of *S.* Newport bacteriophage-resistant mutant in LBM broth cultures challenged with bacteriophages (MOI=1.0) at time 0. Control flask contained cells in LBM buffer without bacteriophage.
Figure 2.6. Fecal counts shed from two calves following challenge with (7.45x10^9 CFU’s) of the S. Newport bacteriophage-resistant mutant strain on Day 0.
Figure 2.7. Fecal *S*. Newport bacteriophage-resistant mutant strain counts shed from two calves following challenge with $1.47 \times 10^{10}$ CFU’s *S*. Newport mutant strain on Day 0.
Figure 2.8. Fecal S. Newport bacteriophage-resistant mutant and S. Newport parent strain counts shed from calf 5 following challenge with $8.5 \times 10^9$ CFU’s of the parent S. Newport strain and $1.11 \times 10^{10}$ CFU’s of the bacteriophage-resistant mutant on Day 0. Body temperature was measured following challenge on Day 0.
Figure 2.9. Fecal S. Newport bacteriophage-resistant mutant and S. Newport parent strain counts shed from calf 6 following challenge with $8.5 \times 10^9$ CFU’s of the parent S. Newport strain and $1.11 \times 10^{10}$ CFU’s of the bacteriophage-resistant mutant on Day 0. Body temperature was measured following challenge on Day 0.
(A) *Salmonella* uses its virulence factors (T3SS-1 and T3SS-2) to elicit host responses that eliminate microbiota bacteria that limits pathogen expansion. T3SS-1-mediated invasion and T3SS-2-mediated survival in tissue induces recruitment of neutrophils. Neutrophils release of reactive oxygen species (ROS) and ROS oxidize the respiratory by-product thiosulphate ($S_2O_3^{2-}$) (which is generated by the microbiota) into tetrathionate ($S_4O_6^{2-}$), which can be used by *Salmonella* spp (but not the microbiota) for respiration. *Salmonella* uses the tetrathionate as a terminal electron acceptor by a tetrathionate reductase to drive a respiratory luminal expansion of *Salmonella* growth. (B) The phage-resistant *Salmonella* is not able to produce the T3SS-1 injectisome, so epithelial invasion is unsuccessful. No host immune response is elicited and the salmonellae is unable to compete against the limiting microbiota. (C) The phage-resistant *Salmonella* is not able to produce the T3SS-1 injectisome, but the parent strain of *Salmonella* is able to invade epithelium and induces inflammation. Although unable to invade, the phage-resistant mutant of *S. Newport* could utilize tetrathionate as a terminal electron acceptor to outcompete the microbiota as long as the tetrathionate reductase is not compromised. The parent and phage-resistant *Salmonella* utilizing tetrathionate as a terminal electron acceptor by the tetrathionate reductase and by the parent strain invades, which helps the bacteriophage-resistant mutant of *S. Newport* to outcompete the microbiota in the intestinal lumen.
2.9. Chapter 2 Appendices

Appendix 2.1. Transmission electron micrographs of S11, S40, S41, S44, S50. Stained with 2% phosphotungstic acid.

Five bacteriophage isolates (S11, S40, S41, S44, S50) originally isolated from Salmonella-containing diagnostic cultures that had been characterized by Hyland et al were used in this study (Hyland et al., unpublished). These five phages show lytic activity against S. Newport in vitro and have shown therapeutic effects in vivo in a S. Newport infection calf model (Hyland et al., unpublished). All five phages belong to the viral order Caudovirales. Phages were placed in their respective family based on morphology (Ackermann, 2007): phage S11 belongs to Podoviridae; S40 and S44 belonging to Siphoviridae; S41 and S50 belonging to Myoviridae.
Appendix 2.2. The average temperature and CFU/g of the *Salmonella* Newport parent strain shed from feces of control calves (n=6) following challenge with \( \approx 5.0 \times 10^9 \) CFU’s of the parent strain on Day 0 (Hyland et al., unpublished).
Chapter 3

Factors Influencing Prevalence of *Salmonella* in a Multi-Species Animal Facility

3.1. Abstract

Diseases caused by serotypes from the bacterial genus *Salmonella* have a major impact on animal and human health. Studies have been conducted to assess factors that contribute to the prevalence of certain *Salmonella* serovars on single-species food animal production operations. Little research has been performed to examine the factors contributing to *Salmonella* serotype incidence in multi-species animal production facilities such as veterinary teaching hospitals, or the ability of *Salmonella* serovars to move to adjacent facilities. We hypothesize that specific factors increase the likelihood of isolating environmental *Salmonella* serotypes in these locations. Information from this study can be used to develop specific interventions to reduce the incidence and movement of *Salmonella* at veterinary schools as well as farms hosting two or more food animal species.

Over a two-year period, 631 environmental samples were collected from various large animal facilities and pastures within a veterinary college. Data was recorded to assess factors that contribute to increased incidence of *Salmonella* contamination. Samples were processed for *Salmonella* isolation according to published protocols, and *Salmonella* isolates were submitted to the National Veterinary Services Laboratories, Ames, IA, or Biovet, Inc., for serotyping. Data was analyzed with Statistical Analysis System (SAS).

Of the 631 samples obtained, 230 (36%) were positive for at least one *Salmonella* serotype. *Salmonella* was recovered from the majority of facilities and areas sampled. Two cattle-associated serotypes, *Salmonella* Muenster (S. Muenster) and/or *Salmonella* Cerro (S. Cerro), were isolated from most facilities and multiple sites across multiple seasons regardless of resident animal species. The factors shown to be significant for isolation using Fisher’s Exact Test for bivariate analysis were season (p=0.008), resident species (p=0.008), and environment (p=0.05). The highest number of *Salmonella*-containing samples was recovered during the warmer seasons and from areas exposed to dairy cattle. *Salmonella* isolates were recovered more
frequently from man-made animal facilities compared to other pastures. Risk ratios of the variables associated with isolation of *Salmonella* serovars included summer season, water and drain samples, samples exposed to cattle, indoor environments such as buildings, and the dairy and food animal barn locations.

The significantly increased frequency of *Salmonella* isolation from environmental samples exposed to dairy cattle indicates that this species is the source of this pathogen, and is serving as an amplifying host for *Salmonella*. The isolation of *S. Cerro* and/or *S. Muenster* from cattle in the vet school dairy herd supports this conclusion.

### 3.2. Introduction

Environmental *Salmonella* contamination can pose a risk to livestock (Ewart et al., 2001; Pandya et al., 2009). Large animals can be reservoirs for transmitting *Salmonella* spp to other animals (Schott et al., 2001). Biosecurity and risk factor analysis of *Salmonella* incidence derived from sampling can help determine areas for interventions to help control *Salmonella* on farms (Fossler et al., 2005; Funk and Gebreyes, 2004). Fecal contaminated food animal carcasses are the principal source of human food-borne infections (Forshell and Wierup, 2006; Humphrey, 2000). Farms and their environments may become contaminated with *Salmonella* spp following *Salmonella* outbreaks or from colonized animals or environmental contamination (Murray, 2000). Pre-harvest interventions that reduce *Salmonella* spp in feces before the agent has a chance to contaminate carcasses can serve as successful on-farm control strategies, but identifying the routes and sources of infection of food animals is critical to develop these interventions (Humphrey, 2000).

Environmental and animal samples also can be easily collected in the veterinary teaching hospital (Pandya et al., 2009). Preventing and controlling potential nosocomial *Salmonella* infections is an important biosecurity concern in veterinary hospitals. Identification of sources or factors that contribute to environmental prevalence of *Salmonella* spp in the hospital environment could help in controlling the spread of *Salmonella* spp and might translate to on-farm contamination issues.

A surveillance study of a veterinary teaching hospital was conducted to better understand the dynamics of *Salmonella* spp in the environment within a multi-species animal facility. Veterinary teaching hospitals have many different species of animals in close proximity to one another at any given time and are usually designed in an open system format. This environment
can be a potential source and reservoir for the transmission of *Salmonella* spp in a veterinary hospital. In fact, *Salmonella* spp are the most commonly associated agents responsible for nosocomial outbreaks among veterinary teaching hospitals (Benedict et al., 2008; Cherry et al., 2004; Morley, 2002; Schott et al., 2001; Steneroden et al., 2010). Persistence of *Salmonella* spp in the environment has been implicated in the spread of *Salmonella* spp among veterinary patients in many nosocomial outbreaks of salmonellosis (Amavisit et al., 2001; Castor et al., 1989; Dunowska et al., 2007; Schaer et al., 2010; Schott et al., 2001; Ward et al., 2005).

Inpatients pose a greater risk (compared to outpatients) for infectious disease acquisition and spread. Many factors such as dietary changes, antimicrobial treatment, surgery, and compromised immune systems can increase the risks for contracting and/or shedding infectious agents such as *Salmonella* spp. It is critical to identify factors that contribute to the contamination of the hospital environment to reduce the risk from transmission of infectious agents from inpatients to hospitalized animals and hospital personnel (Morley and Weese, 2015).

In this study, we utilized the Auburn University College of Veterinary Medicine (AUCVM) John Thomas Vaughan Large Animal Teaching Hospital (J.T. Vaughan Teaching Hospital) and AUCVM facilities such as Animal Health and Research pastures, model dairy pastures, and an Auburn University Experimental Research Station off-site dairy herd operation to study *Salmonella* spp in the environment. Identifying risk factors involved in environmental *Salmonella* spp contamination in the teaching hospital should help us develop intervention strategies to reduce environmental *Salmonella* spp contamination in other multi-species animal facilities such as farms.

### 3.3. Materials and Methods

#### 3.3.1. Sampling from Facilities

Samples were collected by various methods. Swab samples were taken using sterile cotton tip applicators or gauze. Each swab was pre-soaked in 0.1% sterile buffered peptone water (BPW; Difco Buffered Peptone Water, Becton, Dickinson, and Company, Sparks, MD) before use. Swab samples were placed in Whirl-Pak bags (118-ml) for later processing. Fecal samples collected from the environment were placed in Whirl-Pak bags (532-ml) and transported back to the lab for processing. Feed and hay samples were collected by grab sampling. All samples were collected with clean gloves which were changed between samples.
3.3.2. Sampling from Pastures

Pasture sampling was similar to facility samplings with the exception of fecal samples. Feces were collected from five pat samples which were pooled together to represent the pasture.

3.3.3. Water Sampling

Two methods of water collection were employed. One method employed a 60cc catheter-tip syringe to collect small volumes (50-60mL) of water. This was the primary method for sampling water from animal facilities and pastures which included troughs, bodies of water, and/or standing puddled water. The second method was used to collect larger volumes and utilized a sampling container rinsed three times in the water source prior to collection. One-liter water samples were collected from streams and creeks.

Small volume water samples from animal housing troughs and standing puddled water were culture by adding 50mL of sampled water to 50mL of double concentrated BPW (2xBPW), which diluted the 2xBPW to 1xBPW. This sample was then processed as other samples as described below.

Large volume water samples were cultured using the filter membrane technique by filtering the water through a 0.45 µm membrane filter (Nalgene Analytical Test Filter Funnels, Thermo Fisher Scientific). This filter was then cultured on XLT4 agar for 24-48 hours at 37°C. Characteristic Salmonella colonies, which appear black centered on XLT4 agar, were processed as described below.

3.3.4. Dairy Herd Sampling

A convenience sampling of feces from twelve cows from an on-site dairy herd of thirty-four were collected following routine milking when the animals defecated. The fresh fecal sample was collected into a plastic cup and labeled with the cow number. One-gram of fecal material was added to 50mL of BPW and processed as described below.

3.3.5. Salmonella Culture and Detection

The method used to isolate Salmonella spp from environmental samples was a modified protocol from the USDA/FSIS/OPHS Microbiology Laboratory Guidebook’s Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg, and Catfish Products and Carcass and Environmental Sponges (Rose, 2014). In the pre-enrichment step, BPW was added to the Whirl-Pak bags containing the sample and incubated for 24 hours at 37°C. Pre-enriched samples were inoculated into two enrichment broths: 0.5 mL into Tetrathionate broth tubes.
(Difco Tetrathionate Broth Base, BD) and 0.1 ml into Rappaport Vassiliadis (Rappaport-Vassiliadis Enrichment Broth, Oxoid, Basingstoke, Hants., England,) broth tubes. Tubes were incubated 24 hours at 41°C. Selective isolation of *Salmonella* spp was performed by using Xylose Lysine Agar (Difco XLT4 Agar Base, BD) supplemented with Tergitol 4 (XLT4; Difco XLT4 Agar Supplement, BD). *Salmonella* spp colonies appear characteristically black-centered on XLT4, compared to most other colonies, which are colorless. Enrichment broths were streaked onto XLT4 plates which were incubated for 24-48 hours at 37°C. Four characteristic colonies from these plates were sub-cultured onto a second XLT4 agar plate and incubated for 24-48 hours at 37°C. From these secondary streak plates, one characteristic colony from each of the four sub cultured colonies, was biochemically confirmed to be *Salmonella* spp based on results following inoculation on slants of triple sugar iron agar (TSI; Difco Triple Sugar Iron Agar, BD), lysine iron agar (LIA; BBL Lysine Iron Agar, BD), and urea agar ([BBL Urea Agar Base, BD] and 1.5% [Granulated Agar-Agar, EMD, Gibbstown, NJ]). TSI, LIA, and Urea slants were inoculated in tandem with a single pick from a colony by stabbing the butts and streaking the slants in one operation and incubated for 24 hours at 37°C. *Salmonella* spp on LIA should produce a purple butt with (H₂S-positive) or without (H₂S-negative) blackening of the media. A typical control on TSI should produce a yellow butt and red slant, with (H₂S-positive) or without (H₂S-negative) blackening of the media. A typical control on Urea should produce a yellowing of the media. Presumptive *Salmonella* positive colonies were serologically confirmed with polyvalent serum A-V for *Salmonella* spp. (Difco, BD) and then serogrouped based on serology from *Salmonella* Antiserum Poly Groups A, B, C, D, and E (Difco, BD), followed by further serogrouping using antiserum B, C₁, C₂, D₁, E, or K (Difco, BD). Polyvalent antiserum-positive colonies that did not fall in to one of the tested serogroups were considered “serogroup unknown”, but labeled on the basis of the antiserum poly grouping. Serovar characterization was performed at the National Veterinary Services Laboratory in Ames, IA, or by Biovet Inc., Saint-Hyacinthe, QC, on a subset of isolates representative of each environmental sample set.

3.3.6. **Data Collection and Statistical Analysis**

A data collection form was developed to concisely capture important information about each sample to aid statistical analyses (see Appendix 3.1). Information collected on each sample was consolidated for the purpose of statistical analysis. Statistical analysis was performed to determine *Salmonella* prevalence and was calculated for each set of independent variables:
recent weather, year, season, sample type, resident animal species, environment, and location. Statistical Analysis System software (SAS; version 9.3; SAS Institute Inc., Cary, NC) was used for comparing factors associated with Salmonella isolation. Effects on the dependent variable (Salmonella positive or Salmonella negative) were analyzed using Fisher’s Exact Test for independence with Bonferroni’s correction for multiple comparisons. The Bonferroni’s correction for multiple comparisons adjusted $\alpha$ for a four category comparison to 0.00833 and for a five category comparison to $\alpha=0.00714$, while two category comparisons maintained $\alpha=0.05$.

For purposes of statistical analysis, variables were consolidated to variable categories. Rodents, wild birds, and feral cats was consolidated to the category “Wild Animals” and goats, sheep, pigs, llamas, and alpacas was consolidated to the category “Domestic Large Animals”. The equine barns, Kentucky and Griffin, and the large animal isolation facility was consolidated to the category “Equine Barns”. The Wolfe and Carson barns was consolidated to “Food Animal Barns”. The McClary Dairy Barn and Dairy Parlor as well as the heifer, dairy, and nearby domestic large animal pastures were consolidated to the category “Dairy Barns and Pastures”.

Epi Info 7.1.5 (Centers for Disease Control and Prevention, www.cdc.gov/epiinfo) was used to calculate the Risk Ratio (RR), 95% confidence intervals (CI), and the Mid-P exact $p$-values using a 2x2 contingency tables. The $p \leq 0.05$ (two-tailed) was taken as the level of significance.

### 3.4. Results

The total number of samples collected over a two-year span (2014 – 2015) was 631. Out of 631 samples, 36.45% were positive for Salmonella species. Table 3.1 shows the serovars that were serotyped at each facility over the two-year study. All facilities were positive for at least one season through the course of the study, with the exception of the off-site beef herd. S. Muenster and S. Cerro were the predominate serovars isolated throughout the course of the study. S. Anatum, S. Newport, S. Typhimurium, S. Montevideo, S. Mbandaka, S. Bere, and S. Javiana were additional serovars that were serotyped. Each of these serotypes was found in only one facility for one season with the exception of S. Typhimurium, which was isolated during two seasons and in two facilities. S. Typhimurium was isolated originally in Year 1 Summer from the Wolfe Hospital Barn and the Large Animal Isolation Unit. It was isolated a year later (Year 2 Summer) from the Large Animal Isolation Unit.
S. Muenster predominated in Year one Winter with the exception of S. Cerro being isolated from the Wolfe Hospital Barn. Throughout the course of the study, S. Muenster was regularly isolated from many facilities. Beginning in Year 1 Spring, S. Cerro was regularly isolated from many facilities.

Frequency analysis (Table 3.2) was performed to determine prevalence and percentage of *Salmonella*-positive samples for different variables (year, season, weather, environment, source, species, and facility). Out of 631 samples, 230 (36.45%) of samples were positive for *Salmonella* species. A total of 449 samples were collected from J.T. Vaughan Teaching Hospital (excluding samples from Animal Health and Research Pastures and the off-site beef herd) with a *Salmonella*–positive rate of 50.33%. The dairy barns and pastures were not excluded from this rate due to the traffic and personnel movement between the dairy barns/pastures and the J.T. Vaughan Teaching Hospital.

A Fisher’s Exact Test for independence for bivariate analysis with Bonferroni’s correction was used to evaluate the association of the independent variables with *Salmonella* isolation. Comparisons within independent variables were considered significant if *p*-values were as follows: recent weather (*p* ≤ 0.05), year (*p* ≤ 0.05), season (*p* ≤ 0.00833), sample type (*p* ≤ 0.00714), resident animal species (*p* ≤ 0.00833), environment (*p* ≤ 0.05), and location (*p* ≤ 0.00714). Recent weather (dry weather versus recent rainfall) or year (year 1 versus year 2) were not significant (*p* > 0.05). All other independent variables had a grouping with no significant differences (*p* > 0.05) and therefore were considered equal.

Seasons had two groups: group A (summer, spring, and fall) and group B (spring, fall, and winter). The sample type had three groups: group A (water, drain, and fecal samples), group B (fecal and surface samples), and group C (surface and feed/hay samples). Resident animal species was first consolidated to four species categories: bovine, equine, wildlife (birds, rodents, and feral cats, and domestic large animal (sheep, goats, llamas, and pigs). The four species categories had two groups: group A (wildlife, bovine, and domestic large animal) and group B (domestic large animal and equine samples). Comparison by Fisher’s Exact Test found buildings to be significantly different from pastures (*p* < 0.0001). Samples from each location had three groups: group A (dairy barns/pastures and food animal barns), group B (equine/large animal isolation and animal health), group C (animal health and the off-site beef herd).
The risk ratios (Table 3.3) gave more resolution to factors more strongly associated with isolating *Salmonella* species from environmental samples. Variables not associated with isolation of *Salmonella* were recent weather (dry weather versus recent rainfall) and year (year 1 versus year 2). Variables associated with isolation of *Salmonella* were summer season, water and drain samples, samples exposed to bovine residential animals, indoor environments such as buildings, and the dairy and food animal barn locations. The offsite beef cattle herd was negative for *Salmonella* spp through the entire study.

Results for the isolation of *Salmonella* spp from fecal samples collected from the on-site dairy cow herd can be found in Table 3.4. All 12 cows from which fecal samples were obtained were positive for *Salmonella* spp (100%). Five cows were positive for *S. Muenster* (41.7%), 11 cows were positive for *S. Cerro* (91.7%), and 4 cows were positive for both *S. Muenster* and *S. Cerro* (33.3%).

### 3.5. Discussion

The AUCVM J.T. Vaughan Teaching Hospital, like many other small and large animal veterinary teaching hospitals, has substantial environmental contamination of *Salmonella* spp (Benedict et al., 2008; Cherry et al., 2004; Morley, 2002; Schott et al., 2001; Steneroden et al., 2010). This study differs from other studies of *Salmonella* spp in veterinary teaching hospitals in that it was performed in the absence of an outbreak of clinical disease in university or client animals. We also examined the prevalence of *Salmonella* spp over a two-year time span and analyzed factors that may contribute to contamination of facilities. No other surveillance study of *Salmonella* spp at a veterinary teaching hospital has looked at prevalence for as long a period as the current study. We found similar results to previous studies, which the duration of this current study strengthens findings by previous studies. This study was originally designed to serve as a model for environmental prevalence studies of *Salmonella* spp on how to utilize epidemiological and biostatistical methods to determine factors that may contribute to environmental contamination of *Salmonella* in a multi-species animal facility. Analysis of factors and the fact we could recover *Salmonella* from a selected portion of the dairy herd shows that on-site dairy herds have the potential to be sources for widespread environmental contamination at veterinary teaching hospitals and that interventions need to be developed to ensure patient and personnel safety.
The overall prevalence of *Salmonella* spp positive environmental samples at the J.T. Vaughan Teaching Hospital (excluding samples from Animal Health and Research Pastures and the off-site beef herd) was 50.33% (n=449). This prevalence appears to be high compared to environmental *Salmonella* spp prevalence at other veterinary teaching hospitals. Stenerden et al (2010) reported that 22.9% of environmental samples collected at the James L. Voss Veterinary Teaching Hospital at Colorado State University were positive for *Salmonella* spp with 14.2% of samples containing the outbreak strain, which indicated widespread environmental contamination (Steneroden et al., 2010). An outbreak study at Cornell University found that 0.5% of environmental samples was positive for the outbreak strain (Cummings et al., 2014). A separate outbreak investigation at Michigan State University found 1.24% of environmental samples cultured and 12% of environmental samples PCR tested for the outbreak strain were positive during facility cleaning and disinfection (Schott et al., 2001). At the New Bolton Center at the University of Pennsylvania School of Veterinary Medicine, 3.3% of environmental samples were positive for *Salmonella* spp prior to the outbreak. During an outbreak of *Salmonella* Newport within the large animal veterinary teaching hospital, 30% of environmental samples were positive for *Salmonella* Newport which indicated widespread contamination (Schaeer et al., 2010). Comparing the prevalence found at our veterinary teaching hospital to these other veterinary teaching hospitals with environmental contamination issues, it could be concluded that there is widespread contamination of *Salmonella* spp at the J.T. Vaughan Teaching Hospital at Auburn University.

The sections of highest prevalence (Table 3.2) in our study were the dairy barns/pastures as well as the food animal barns, which had 69% and 60% positive *Salmonella* spp prevalence, respectively. These two were not statistically different from one another (*p* > 0.05), which is not surprising because individuals in these units work within both units. It could be presumed that something as simple as individuals moving between these two facilities may be a source of spread of the *Salmonella* spp. There are currently no footbaths or any other barriers in place to prevent movement of infectious disease agents among the facilities, which may increase the likelihood of individuals moving pathogens from one area to another. The sharp decrease in prevalence between food animal barns and the equine barns may be attributed to the footbaths located at the front and rear of the equine barns and that individuals working in the equine barns do not work in the food animal or dairy barns.
We found that environmental prevalence of *Salmonella* spp was not statistically different ($p > 0.05$) between Summer, Fall, and Spring, but risk ratios (*Table 3.3*) determined that the Summer season was associated with the isolation of *Salmonella* spp. This finding is similar to previous studies in that prevalence of *Salmonella* spp among dairy cattle is higher in the Spring, Summer, and Fall seasons with peaks typically during Summer months (Cummings et al., 2009c; Fossler et al., 2005; Pangloli et al., 2008). Cummings et al (2009c) found Fall to be significantly higher for shedding of *Salmonella* spp in calves admitted to a veterinary teaching hospital (Cummings et al., 2009c). Pangloli et al (2008) found *Salmonella* spp to have a high prevalence ($> 40\%$) in environmental samples in all seasons with the exception of winter (Pangloli et al., 2008). A study of cattle and environmental sampling factors on *Salmonella* spp among dairies found that Fall, Spring, and Summer seasons were factors associated with *Salmonella* shedding in cattle (Fossler et al., 2005). We found no difference between recent rainfall and dry weather regarding prevalence ($p > 0.05$) which is different from previous studies that found prevalence of *Salmonella* to increase with rainfall (Haley et al., 2009; Jacobsen and Bech, 2012; Polo et al., 1999).

It is not surprising that buildings, water sources, and drain sources had the highest prevalence (*Table 3.2*) among the environment (building versus pasture) and sample sources (water, drain, surface, feed/hay, and fecal samples). Water samples and drain swabs were associated with the isolation of *Salmonella* by risk ratio among all the sources. Additionally it was not surprising to find water sources associated with isolation of *Salmonella* spp because water can be a source for dissemination of enteric pathogens to livestock (Doyle and Erickson, 2006). We found an association for isolation of *Salmonella* spp with drain samples, similar to the findings of others who isolated *Salmonella* from floor drains (Castor et al., 1989; Schott et al., 2001; Ward et al., 2005). Others have found that drain surfaces were the most common site of *Salmonella* recovery with a prevalence of 7.3% (Pandya et al., 2009); we also found that the highest prevalence of *Salmonella* was recovered from water and drain sources, but at a much higher level (49%; *Table 3.2*). The multiple comparisons by Fisher’s Exact Test showed that water, drain, and fecal samples are the most important type of samples to collect when actively surveying for environmental *Salmonella*.

Buildings, the dairy barns and pastures, and the food animal barns were all associated with isolation of *Salmonella* spp (*Table 3.3*). This finding and the finding that there was an
association of bovine species with the isolation of *Salmonella* spp lead us to examine the resident dairy cattle herd as the source of contamination. This herd is milked twice daily in the McClary Dairy Barn by students and employees. *Salmonella* Muenster and *Salmonella* Cerro were isolated from twelve cows sampled from the on-site dairy herd (*Table 3.4*), and these were the two major serovars isolated from environmental samples over the two-year span of this study (*Table 3.1*). We speculate that these pathogens originate from the dairy herd and there may be reseeding of the organisms into the environment daily. Considering that *Salmonella* Muenster and *Salmonella* Cerro have been associated with dairy cows previously, this is a plausible scenario (Chapagain et al., 2007; Cummings et al., 2009b; Cummings et al., 2010; Hoelzer et al., 2011; Kessel et al., 2007; Loneragan et al., 2012; Radke et al., 2002; Rodriguez-Rivera et al., 2016, 2014; van Warnick et al., 2003).

The intermittent (versus constant) isolation of *S*. Muenster and *S*. Cerro from the equine barns suggests that the horses in these barns are not the reservoir for these *Salmonella* serovars. Also, the horses housed in these barns are hospital patients so there is a constant turnover within these facilities as new patients are admitted and older patients are discharged.

*S*. Typhimurium was isolated from the Large Animal Isolation facility during both Summer seasons. *S*. Typhimurium is the most commonly isolated serovar from horses (CDC, 2014). This finding, along with the fact that most clinical cases of salmonellosis occur in the summer months, might explain why this facility was positive for this serovar at two time points (Cummings et al., 2009c; Fossler et al., 2005; Pangloli et al., 2008). The good biosecurity and biocontainment practices followed by the personnel in this facility probably prevent these isolates from persisting in the environment.

Environmental surveillance programs for *Salmonella* spp have shown a correlation between environmental contamination and infection in animals (Burgess et al., 2004; Dunowska et al., 2007; Ewart et al., 2001; Schaer et al., 2010; Traverse and Aceto, 2015). Identifying the source of environmental contamination or factors associated with contamination is critical for developing interventions to prevent infections in animals. Hopefully interventions that interrupt transmission from the environment to animals also help prevent movement of zoonotic agents into the food chain at the pre-harvest level.

Our results are similar to other studies which suggest that individuals working within the food animal section (dairy barns/pastures and food animal barns) should be more aware of
potential risks of nosocomial and zoonotic infections by *Salmonella* spp and implement intervention strategies to prevent transmission. The food animal section workers should have training in good hygiene, biosecurity, and disease control programs. A good resource which describes the general principles of an infectious disease control program in large animal veterinary hospitals is available (Smith et al., 2004). Currently at the large animal teaching hospital, minimal standard operating procedures exist for the monitoring and cleaning practices to contain or prevent *Salmonella* spp contamination.

There is no “one size fits all” infection control and prevention program. An appropriate infection control plan should be tailored to a facility’s unique operational limits (Burgess and Morley, 2015; Traub-Dargatz et al., 2004). This study found *Salmonella* associated with dairy barns/pastures and the dairy herd, but this may not be same situation at all institutions. *Salmonella* has been documented to move from equine facilities to non-equine patients at Cornell University and the University of Pennsylvania veterinary teaching hospitals (Cummings et al., 2014; Schaer et al., 2010). Analysis of the AUCVM teaching hospital indicates critical control points involving our on-site dairy herd that are unique to the AUCVM. The AUCVM is fortunate to have a model dairy to train students and analysis from this study should help individuals within these areas to remain vigilant in the prevention of transmission of infectious agents such as *Salmonella*.

This study is part of a larger investigation aimed at examining the dynamics of *Salmonella* movement among different animal species raised in proximity to one another. Future work will include the utilization of pulse-field gel electrophoresis to determine if the same or different strains of *S*. Muenster and *S*. Cerro are present in positive sample sites across time. These results will help us determine if movement is occurring. A future study that could be beneficial is for the completion of the serovar analysis on all *Salmonella* positive isolates to analyze risk factors at a *Salmonella* serovar level. This way we would be able to determine environmental risk factors of the serovars *S*. Muenster and *S*. Cerro.

3.6. Conclusions

The intermediate occurrence of *Salmonella* serovars recovered in the equine barns, would suggest that the source of this contamination is an external source to these barns. The significantly increased frequency (*p* < 0.05) of *Salmonella* isolation from environmental samples exposed to dairy cattle suggests that this species is either the source of this pathogen, or is
serving as an amplifying host for \textit{Salmonella}. Recovery of \textit{Salmonella} from all the sampled cows and the fact that both \textit{S.} Muenster and \textit{S.} Cerro are associated with cattle supports this conclusion. This study is the first reported long-term surveillance study of \textit{Salmonella} at a veterinary teaching hospital.

3.7. References


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Table 3.1. *Salmonella* serovars isolated from units associated with Auburn University College of Veterinary Medicine over the time-span of two years.

<table>
<thead>
<tr>
<th>Facility</th>
<th>Year 1 Winter</th>
<th>Year 1 Spring</th>
<th>Year 1 Summer</th>
<th>Year 1 Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equine - Griffin Barn¹</td>
<td>S. Muenster</td>
<td>Negative</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>Equine - Kentucky Barn¹</td>
<td>S. Muenster</td>
<td>Negative</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>Large Animal Isolation Facility¹</td>
<td>Negative</td>
<td>Negative</td>
<td>S. Typhimurium</td>
<td>Negative</td>
</tr>
<tr>
<td>Wolfe Barn - Food Animal Barn²</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Typhimurium</td>
</tr>
<tr>
<td>Carson Barn Food Animal Barn²</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>McClary Barn-Dairy Hospital Barn³</td>
<td>S. Muenster</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
</tr>
<tr>
<td>McClary Barn-Dairy Parlor³</td>
<td>S. Muenster</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
</tr>
<tr>
<td>Dairy Cow/Heifer Herd Road³</td>
<td>S. Muenster</td>
<td>S. Muenster</td>
<td>S. Muenster</td>
<td>S. Newport</td>
</tr>
<tr>
<td>Central Hay Barn³</td>
<td>S. Muenster</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
</tr>
<tr>
<td>Calf Fields³</td>
<td>S. Muenster</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
</tr>
<tr>
<td>Goat Pens³</td>
<td>No Samples Collected</td>
<td>S. Muenster</td>
<td>S. Muenster</td>
<td>No Samples Collected</td>
</tr>
<tr>
<td>Lab Animal Health Pastures⁴</td>
<td>S. Muenster</td>
<td>S. Muenster</td>
<td>S. Anatum</td>
<td>Negative</td>
</tr>
<tr>
<td>Off-Site Beef Herd²</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

¹Equine Barns and Large Animal Isolation
²Food Animal Barns
³Dairy Barns and Pastures
⁴Animal Health and Research Pastures
⁵Off-Site Beef Herd
Table 3.1(Continued). *Salmonella* serovars isolated from units associated with Auburn University College of Veterinary Medicine over the time-span of two years.

<table>
<thead>
<tr>
<th>Facility</th>
<th>Year 2 Winter</th>
<th>Year 2 Spring</th>
<th>Year 2 Summer</th>
<th>Year 2 Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equine - Griffin Barn¹</td>
<td>Negative</td>
<td>S. Cerro</td>
<td>Negative</td>
<td>S. Muenster</td>
</tr>
<tr>
<td>Equine - Kentucky Barn¹</td>
<td>Negative</td>
<td>Negative</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>Large Animal Isolation Facility³</td>
<td>Negative</td>
<td>Negative</td>
<td>S. Typhimurium</td>
<td>Negative</td>
</tr>
<tr>
<td>Wolfe Barn -Food Animal Barn²</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Mbandaka</td>
<td>S. Muenster</td>
</tr>
<tr>
<td>Carson Barn Food Animal Barn²</td>
<td>Negative</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>McClary Barn-Dairy Hospital Barn¹</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>McClary Barn-Dairy Pafo²</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>Dairy Cow/Heifer Herd Road³</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>Central Hay Barn¹</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>Calf Fields³</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>Goat Pens³</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
<td>S. Muenster</td>
<td>S. Cerro</td>
</tr>
<tr>
<td>Lab Animal Health Pastures⁴</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Off-Site Beef Herd⁵</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

¹Equine Barns and Large Animal Isolation  
²Food Animal Barns  
³Dairy Barns and Pastures  
⁴Animal Health and Research Pastures  
⁵Off-Site Beef Herd
Table 3.2. Frequency analysis of factors to determine prevalence of *Salmonella*-positive status from environmental samples.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Levels</th>
<th><em>Salmonella</em>-Positive / Total Environmental Samples</th>
<th>Percentage of Samples <em>Salmonella</em>-Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>One</td>
<td>137 / 349</td>
<td>39%</td>
</tr>
<tr>
<td></td>
<td>Two</td>
<td>93 / 282</td>
<td>33%</td>
</tr>
<tr>
<td>Season</td>
<td>Fall</td>
<td>47 / 134</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>52 / 143</td>
<td>36%</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>89 / 188</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>42 / 166</td>
<td>25%</td>
</tr>
<tr>
<td>Weather</td>
<td>Dry Weather</td>
<td>101 / 295</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>Recent Rainfall</td>
<td>129 / 336</td>
<td>38%</td>
</tr>
<tr>
<td>Environment</td>
<td>Building</td>
<td>171 / 356</td>
<td>48%</td>
</tr>
<tr>
<td></td>
<td>Pasture</td>
<td>59 / 275</td>
<td>21%</td>
</tr>
<tr>
<td>Source</td>
<td>Drain</td>
<td>50 / 102</td>
<td>49%</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>69 / 188</td>
<td>37%</td>
</tr>
<tr>
<td></td>
<td>Feed/Hay</td>
<td>20 / 113</td>
<td>18%</td>
</tr>
<tr>
<td></td>
<td>Surface</td>
<td>28 / 100</td>
<td>28%</td>
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<td></td>
<td>Water</td>
<td>63 / 128</td>
<td>49%</td>
</tr>
<tr>
<td>Species</td>
<td>Bovine</td>
<td>189 / 429</td>
<td>44%</td>
</tr>
<tr>
<td></td>
<td>Domestic Large Animal</td>
<td>12 / 44</td>
<td>27%</td>
</tr>
<tr>
<td></td>
<td>Equine</td>
<td>17 / 129</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>Wildlife</td>
<td>12 / 29</td>
<td>41%</td>
</tr>
<tr>
<td>Facility</td>
<td>Food Animal/Beef Barns</td>
<td>48 / 80</td>
<td>60%</td>
</tr>
<tr>
<td></td>
<td>Dairy Barns and Pastures</td>
<td>159 / 232</td>
<td>69%</td>
</tr>
<tr>
<td></td>
<td>Equine Barns and Large Animal Isolation</td>
<td>19 / 137</td>
<td>14%</td>
</tr>
<tr>
<td></td>
<td>Animal Health and Research Pastures</td>
<td>4 / 80</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>Off-Site Beef Herd</td>
<td>0 / 102</td>
<td>0%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>230 / 631</td>
<td>36%</td>
</tr>
<tr>
<td>Variable</td>
<td>Levels</td>
<td>Risk Ratio</td>
<td>95% Confidence Interval</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------</td>
<td>------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>Year</td>
<td>One</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Two</td>
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<td>0.7</td>
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<tr>
<td>Season</td>
<td>Summer*</td>
<td>1.5</td>
<td>1.2</td>
</tr>
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<td></td>
<td>Spring</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Fall</td>
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<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
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<td>0.5</td>
</tr>
<tr>
<td>Weather</td>
<td>Recent Rainfall</td>
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<td>0.9</td>
</tr>
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<td></td>
<td>Dry Weather</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Environment</td>
<td>Building*</td>
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<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Pasture</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Source</td>
<td>Water*</td>
<td>1.5</td>
<td>1.2</td>
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<tr>
<td></td>
<td>Drain*</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
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<td>0.8</td>
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<tr>
<td></td>
<td>Feed/Hay</td>
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<td>0.5</td>
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<tr>
<td></td>
<td>Surface</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Species</td>
<td>Bovine*</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Wildlife</td>
<td>1.1</td>
<td>0.7</td>
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<tr>
<td></td>
<td>Domestic Large Animal</td>
<td>0.7</td>
<td>0.4</td>
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<td></td>
<td>Equine</td>
<td>0.3</td>
<td>0.2</td>
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<tr>
<td>Facility</td>
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<td>3.9</td>
<td>3.1</td>
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<td></td>
<td>Food Animal/Beef Barns*</td>
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<td></td>
<td>Equine Barns and Large Animal Isolation</td>
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<td>Animal Health and Research Pastures</td>
<td>0.1</td>
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</tr>
<tr>
<td></td>
<td>Off-Site Beef Herd</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Variable levels associated with risk of isolating *Salmonella* spp.
Mid-P exact $p$-value significance does not associate risk with isolation of *Salmonella* spp, but rather is indicative of factors associated with or associated against isolation of *Salmonella* spp.
Table 3.4. Dairy Cow Herd sampling of feces for the presence of *Salmonella* spp.

<table>
<thead>
<tr>
<th>Dairy Cow Number</th>
<th>Salmonella Serovar (+/-)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. Muenster</td>
</tr>
<tr>
<td>108</td>
<td>+</td>
</tr>
<tr>
<td>119</td>
<td>-</td>
</tr>
<tr>
<td>210</td>
<td>-</td>
</tr>
<tr>
<td>216</td>
<td>-</td>
</tr>
<tr>
<td>217</td>
<td>+</td>
</tr>
<tr>
<td>220</td>
<td>+</td>
</tr>
<tr>
<td>301</td>
<td>+</td>
</tr>
<tr>
<td>302</td>
<td>+</td>
</tr>
<tr>
<td>307</td>
<td>-</td>
</tr>
<tr>
<td>908</td>
<td>-</td>
</tr>
<tr>
<td>1001</td>
<td>-</td>
</tr>
<tr>
<td>1004</td>
<td>-</td>
</tr>
</tbody>
</table>

a +, dairy cow positive for *Salmonella* serovar; - dairy cow negative for *Salmonella* serovar
3.9. Chapter 3 Appendices

Appendix 3.1. Environmental Sample Form to collect information on each individual sample.
Chapter 4. Discussion and Future Directions

4.1. Introduction

The work described in this thesis examined two very different areas of the microbiology of Salmonella. The first area dealt with a bacteriophage-resistant Salmonella mutant in the context of the usefulness of bacteriophage treatment as a novel approach to reduce Salmonella in a calf infection model. The second area examined the epidemiology of Salmonella in a mixed-animal species environment. The main overlap between the two studies was their focus on the Salmonella as a zoonotic foodborne pathogen, and the potential for developing impact on pre-harvest food safety interventions.

4.2. A Study of a Bacteriophage-Resistant Salmonella Mutant

The use of bacteriophages (phages) as a novel approach to reduce foodborne pathogens could lead to the unintended consequence of generating bacteriophage-resistant mutants of these pathogens. Others have evaluated phage-resistant mutants in animal models such as mice, but little work has been done to evaluate phage-resistant mutants in calves (Capparelli et al., 2010a, 2010b; Laanto et al., 2012; Santander and Robeson, 2007; Shamim Hasan Zahid et al., 2008; Smith and Huggins, 1983). The focus of this study was to isolate a phage-resistant mutant of Salmonella Newport, characterize the mutant, and determine if the phage-resistance confers hypervirulence or attenuation.

Isolating a phage-resistant mutant from Salmonella-infected calves which have been treated with bacteriophages is very difficult due to the small number of colonies recovered from feces. This is why the mutant was selected from an overnight culture of phage sensitive S. Newport. The phage-resistant mutant selected showed resistance to four out of five of the phage in the cocktail used by Hyland et al. in their study (Hyland et al., unpublished). The mutation appeared very stable after twenty passages, i.e., it maintained its phage resistant phenotype, and it appeared that the mutation did not decrease fitness from in vitro experiments in broth (Fig. 2.3, Chapter 2). The mutation appears to affect the expression or confirmation of a molecule on the
surface of the resistant mutant, since the phage was no longer able to adsorb to the surface of the bacteria as shown from the adsorption constants.

Two oral challenges with different doses of the phage-resistant mutant indicated that the phage-resistant mutation is attenuated in the calves. The calves did not display any clinical disease and fecal shedding of the phage-resistant mutant dropped rapidly after oral challenge. The reduced fecal shedding of the bacteriophage-resistant *S.* Newport mutant compared to the parent strain in the first two experiments suggests that the mutation(s) may have hindered its ability to colonize the host. Previous experiments with the phage-sensitive parent strain showed that on average, the parent shed countable numbers in the feces for 12 days (Hyland et al., unpublished). The mutant strain, however, was cleared from calf 1 to below our detection levels by day 4 and from calf 2 by day 3 (Fig. 2.8, Chapter 2). The calves receiving the higher inoculum of the mutant displayed slightly different results. Calf 3 cleared the mutant strain below our detection levels by day 8 and calf 4 by day 10 (Fig. 2.9, Chapter 2). No disease signs were observed during periods of shedding or post-shedding of the mutant strain by either pair of calves. We speculate that virulence has decreased in the phage-resistant mutant strain due to this strain’s compromised fitness.

The competition experiment involving co-inoculation of the parent and phage-resistant strains of *S.* Newport into a pair of calves, however, led to unexpected results. We expected the mutant to be cleared rapidly as it was when challenged singly into calves, while the parent would show countable numbers in the feces for ≥12 days and the calves would show disease signs. Instead, both calves 5 and 6 had prolonged shedding of both parent and mutant *S.* Newport strains. One could speculate that the higher inoculation dose of the combined parent and phage-resistant strains of *S.* Newport somehow overcame the attenuating effects of mutation in the mutant, but we have no control experiments with this high dose of parent strain with which to compare. The cause of this higher, prolonged shedding is not fully understood at this time. More studies with additional calves will be conducted to determine the reproducibility of this work and to answer follow-up questions.

We do not know the location of the mutation responsible for the phage-resistance phenotype. Although our adsorption kinetics data indicates that the mutant has lost the receptor(s) for phage binding, just knowing this is not enough to determine the location of the mutation. Many extracellular components, including cell envelope proteins, sugar moieties
including components of the LPS, or other cell envelope moieties like flagella, pili, and capsules can serve as phage receptors. Mutations in the genes for any of these components could generate the phage-resistant phenotype by altering these structures, but other mutations could also affect the phenotypic expression of these potential receptors (Cota et al., 2015). For instance, anything that might alter the translocation of lipopolysaccharide or outer membrane proteins could affect the expression of these structures onto the surface of the cell (Burmann et al., 2015; Hardie et al., 1996; Islam and Lam, 2014; Ruiz et al., 2006). Whole genome sequencing of the parent and mutant strains, followed by sequence alignment and analysis, should help us identify the mutation (Duerkop et al., 2016).

Based on the limited information we have available from our experiments; we hypothesize that the mutation may affect the expression of the type III secretion system-one (T3SS-1). The T3SS-1 is essential for Salmonella invasion of the gut epithelium (Keestra-Gounder et al., 2015; Nieto et al., 2016; Que et al., 2013; Ryu et al., 2014). Once Salmonella invades a host, an immune cascade occurs that results in the production of reactive oxygen species that converts thiosulfate to tetrathionate (Hensel et al., 1999). Salmonella can then utilize tetrathionate as a terminal electron acceptor for respiration to out-compete the intestinal microbiota (Bäumler et al., 2011; Hallstrom and McCormick, 2011; Rivera-Chávez and Bäumler, 2014; Winter et al., 2010). This may be the reason that the phage-resistant mutant, which in our hypothetical model would not invade and generate tetrathionate in the calf gut, was not able to persist in the calves when given alone. However, introducing the parent that is able to invade would result in the cascade of reactions leading to tetrathionate formation. When tetrathionate is formed in the intestinal lumen, the parent and phage-resistant strains are then both able to utilize it to out-compete the intestinal microbiota. One way to test the hypothesis that the phage-resistant mutant is not able to invade is to perform an invasion assay in cell culture (Lockman and Iii, 1990; Pfeifer et al., 1999; Stone et al., 1992). A gentamicin protection assay can distinguish if a mutant is defective in attachment or in invasion. This assay involves a monolayer of mammalian cells incubated with bacterial cells allowing binding and invasion to occur. Samples are divided into three sets; the first set lyses host cells with a mild detergent and the solution is plated on agar plates to determine total colony forming units. The second set involves washing host cells to remove unattached bacterial cells, then host cells are lysed and plated on agar plates to determine attachment. Finally, the third set has gentamicin, an antibiotic
that does not penetrate the mammalian cells, added to a culture to kill extracellular bacteria. The solution is washed of gentamicin, host cells are lysed, and plated on agar to determine invasion (Wilson et al., 2011).

The generation of a multi-bacteriophage-resistant mutant of S. Newport following exposure to a single bacteriophage suggests that the phage resistant strain acquired a mutation(s) that prevents phage adsorption. We hypothesize that phages S11, S40, S41, and S50 all adsorb to the same S. Newport receptor, while S44 adsorbs to a different receptor. It also appears that the mutation which confers bacteriophage-resistance has attenuated the mutant when compared to the parent individually in our calf experimental model of salmonellosis.

4.3. Factors Influencing Prevalence of Salmonella in a Multi-Species Animal Facility

The initial aim of this study was to determine if proximal movement of Salmonella spp occurs within a multi-species animal facility. Our original intention was to use a veterinary teaching hospital campus, which houses multiple animal species in close proximity to one another, to model how proximal movement of Salmonella spp might occur on a farm with multiple animals, such as farms with both cattle herds and chicken flocks. The goal was to delineate patterns of Salmonella spp movement between animal species in order to develop interventions to reduce Salmonella burden among food producing animals.

The first step towards fulfilling the study aim was to collect environmental samples from the Auburn University College of Veterinary Medicine campus. The off-site beef herd was added due to the fact that individuals working within the J.T. Vaughan Large Animal Teaching Hospital periodically travel to the off-site beef herd. Samples were collected quarterly over a two-year time span (Table 3.1, Chapter 3). After all of the samples had been collected, statistical analysis of data collected for each sample was performed. A limitation of the study was that statistical analysis was limited to bivariate analysis of Salmonella positive vs Salmonella negative. It would have been informative to determine the serovars of all isolates collected from the 230 samples, but limited funds restricted the number of isolates that could be serotyped. To select isolates to send off for serotyping, isolates from each facility were serogrouped based on location and one isolate from each serogroup was sent to the NVSL for serotyping. Most of the isolates were found to be either Salmonella serogroup E or Salmonella serogroup K. The subset of the group E isolates which were serotyped were S. Muenster, while the subset of the group K isolates was S. Cerro. All additional isolates that did not react with the
group E or K antisera, such as the serogroup B or serogroup C2 isolates, were sent off for serotyping. One could speculate that all the non-serotyped *Salmonella* serogroup E or *Salmonella* serogroup K isolates would be *S.* Muenster (serogroup E) and *S.* Cerro (serogroup K). Further studies could include the serotyping of all additional isolates in order to show factors associated with the isolation of *Salmonella* based on serovars.

This study is an example of conducting environmental surveillance, identifying factors associated with isolation of a pathogen, and then use this information to identify a potential source. After conducting a hazard analysis and identifying critical control points in the system (Morley, 2002), the next step of the study would be to implement interventions to reduce environmental contamination with *Salmonella*. Restriction of human and patient movement between the McClary Dairy Barns and the Dairy Pastures, and regular environmental sanitation could assist with reduction of the environmental *Salmonella* burden (Burgess and Morley, 2014; Morley and Weese, 2015). Veterinary infection control specialists agree that one of the most important steps in infection control in veterinary care settings is educating veterinary personnel of the risks related to nosocomial and zoonotic infections (Ekiri et al., 2010; Morley, 2013; Traub-Dargatz et al., 2004). Therefore, interventions should involve biosecurity and infection control formal training for all employees and students within the Food Animal section (Morley and Weese, 2015; Pandya et al., 2009). After instituting intervention/education steps, we recommend regular long-term environmental monitoring of the occurrence of environmental contamination with specific pathogens of concern.

A novel intervention strategy might be to utilize *Salmonella*-targeted bacteriophage(s) as a surface disinfectant inside McClary Dairy Barns. Twice daily milking of the dairy cow herd represents an ongoing source of fecal contamination. The lytic activity of bacteriophages sprayed on the surfaces of the dairy barns would be expected to decrease low-level environmental *Salmonella* contamination that surveillance data indicates is present and to reduce the likelihood of spread to the rest of the veterinary campus. Utilizing bacteriophages as part of a program to control the bioburden of pathogens on environmental surfaces has been reported by several research groups (Chen et al., 2013; Dancer, 2014; Mahony et al., 2011; Page et al., 2009; Sharma et al., 2005; Tomat et al., 2014; Viazis et al., 2011). In fact, wild bacteriophages targeting *S.* Muenster and *S.* Cerro have been isolated and characterized in our laboratory from some of the environmental samples in this study.
Of the isolates serotyped, the serovars *S. Muenster* and *S. Cerro* predominated (Table 3.1, Chapter 3). *S. Muenster* contamination appeared widespread throughout the study. *S. Cerro* was initially isolated from just one facility (Wolfe Barn – Food Animal Barn), however over the course of the study, this serovar has been isolated at adjacent buildings/facilities. And we cannot rule out the possibility that multiple strains of *S. Cerro* (and *S. Muenster*) have been circulating in the vet school environment prior to our recognition of them in this environment. We could test this experimentally by utilizing one or more subtyping methods to identify individual strains of *S. Muenster* or *S. Cerro*. Possible methods include pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism-polymerase chain reaction, ribotyping, random amplification of polymorphic DNA, arbitrarily primed polymerase chain reaction, variable number tandem repeat analysis, single locus sequence typing, multilocus sequence typing, and whole-genome sequencing (WGS) (Abatcha et al., 2014; Boccia et al., 2015; Foley et al., 2009, 2007; Sabat et al., 2013; Wattiau et al., 2011). If the same strain of *S. Cerro* is isolated from different sites over time, it would lead us to conclude that proximal movement was occurring.

Of the subtyping methods available, the two in most use now are PFGE and WGS (Carleton and Gerner-Smidt, 2016). PFGE is considered the “gold standard” for strain typing of bacterial pathogens and a standardized technique used by the National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet) (Bopp et al., 2016; Kérouanton et al., 2007; Wattiau et al., 2011). PFGE provides enough discrimination among bacterial isolates that it is being evaluated for *Salmonella* serotyping in state public health laboratories (Bopp et al., 2016; Kérouanton et al., 2007). WGS is the more promising of these two techniques due to the fact that next-generation sequencing technology is drastically reducing the cost and time needed to sequence bacterial genomes and it allows the possibility of identification of virulence factors and antimicrobial resistance factors. PulseNet is testing WGS and it is beginning to replace PFGE as the new gold standard for subtyping (Carleton and Gerner-Smidt, 2016; Salipante et al., 2015). WGS analysis can either compare single-nucleotide polymorphisms or make gene-by-gene comparisons with whole-genome multilocus sequence typing (wgMLST). Public health laboratories, food regulatory laboratories, and universities are assisting PulseNet in building a standardized WGS database for WGS single-nucleotide polymorphisms analysis or wgMLST (Carleton and Gerner-Smidt, 2016).
Overall, this project found that season, resident animal species, sample source, and the facility are factors which affect the chances of recovering *Salmonella* spp from the environment in a multi-species animal facility. Our data suggest that *Salmonella* is able to move to proximal locations and establish a presence in animal facilities at a distance from the source. Although one or more specific mechanisms remains to be elucidated, *S. Cerro* moved beyond its initial isolation at a food animal hospital barn to adjacent dairy and equine facilities, and then beyond these enclosures to nearby cattle pastures and a hay barn. Once established in an area, *Salmonella* appears to either maintain itself or continually be re-seeded into that area. The significantly increased frequency ($p < 0.05$) of *Salmonella* isolation from environmental samples associated with dairy cattle, along with the well-recognized association of both *S. Muenster* and *S. Cerro* with cattle, suggested to us that the dairy herd was either the source of this pathogen or is serving as an amplifying host for *Salmonella*. When fecal culture of 12 dairy herd cows was performed, either *S. Muenster* or *S. Cerro*, or both, were isolated from all 12 animals, confirming the dairy herd as the probable source of the contamination.

### 4.4. References


Chapter 5. References


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