Bovine salmonellosis and the challenge of developing cross protective vaccines for this disease

by

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Abstract

Salmonella contamination of meat is a leading cause of foodborne illness around the world. Nontyphoidal *Salmonella* are responsible for an estimated 94 million infections and 155,000 deaths worldwide each year. Of these infections, 86% are estimated to be foodborne. Infection of dairy and beef cattle can lead to contamination of milk and milk products as well as processed beef. Once cattle are infected, *Salmonella* can be found in many organs of the animals. Peripheral lymph node infections are of particular interest, because these lymph nodes along with hides are the main culprits of meat contamination during processing.

Vaccination of production food animals is one of several strategies of prevention and control of *Salmonella* infections and outbreaks. Vaccination is becoming even more important with the reduction of prophylactic antibiotic use that is driven by an increase in antibiotic resistant bacteria isolated from a variety of food production animals. There are limited commercially available vaccines for cattle that have shown effectiveness, but great strides are being made in this area of research. The vast number of *Salmonella* serovars with differences in vital virulence factors capable of infecting cattle makes developing vaccines that are cross protective very difficult. This report discusses the known virulence factors of *Salmonella*, the disease symptoms of bovine salmonellosis, prevention and control strategies, and the development of new vaccines.

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Acknowledgements

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Finally, I would like to express immeasurable thanks to my wonderful wife, Laura Engels, for all her love and encouragement. She has been so unbelievably supportive of my goal these last few years, and I am extremely grateful for that. Laura makes me complete, and I eagerly await the next chapter in our lives together.

Dedication

This report is dedicated to my wife, Laura, and our children, Jamie, Bradley, and Daniel.

Chapter 1 - Introduction to *Salmonella* and Bovine Salmonellosis

Salmonella enterica spp. e*nterica* (*Salmonella*) is a genus of Gram-negative bacteria comprised of non-spore forming bacilli belonging to the *Enterobacteriacea* family (Coburn *et al*., 2007; Dunkley *et al*., 2009; Agbaje *et al*., 2011). The genus is comprised of two species, as described below (Reeves *et al*., 1989; Su and Chiu, 2007), and they are characterized by motility mediated by peritrichous flagella, and facultative anaerobic metabolism (Coburn *et al*., 2007; Agbaje *et al*., 2011; Andino and Hanning, 2014). Like other *Enterobacteriacea*, *Salmonella* live in the gastrointestinal tract, often without evidence of clinical disease, of many mammals, birds, reptiles and fish, but also *Salmonella* can persist in the environment (Sanchez *et* al., 2002; Callaway *et al*., 2014). The genus is named after D.E. Salmon who, along with Theobald Smith discovered and isolated "bacillus cholerasuis" (now known as *Salmonella* Choleraesuis) from porcine intestines in 1884 (Agbaje *et al*., 2011). *Salmonella* nomenclature is very complex and there have been many changes in conventional naming schemes throughout the history of the genus (Salyers 2002; Bopp *et al*., 1999). Originally, salmonellae were separated into different "serovars" (also called "serotypes") according to a scheme of serological classification of poly-O (cell wall) and H (flagellar) antigens described by Kaufman and White, who also proposed that each serovar be considered a separate species (Brenner *et al*., 2000). Currently, there are 67 O-antigens and 117 H-antigens that have been identified (Grimont and Weill, 2007; Popoff, 2001). According to the current Centers for Disease Control and Prevention (CDC) system, the genus *Salmonella* consists of two species: *Salmonella enterica* and *Salmonella bongori* (Su and Chiu, 2007). Within *Salmonella enterica*, there are six subspecies: *enterica*, *salamae, arizonae, diarizonae, houtenae,* and *indica* (Figure 1-1) (Su and Chiu, 2007). *Salmonella enterica* spp. *enterica* is most relevant to animal disease and responsible for 99% of human *Salmonella*

infections (Uzzau *et al.*, 2000). *Salmonella enterica* spp. *enterica* can be further classified into serovars (Fierer and Guiney, 2001) and more than 2,600 serovars have been described (Table 1-1) (Grimont and Weill, 2007; Hendriksen *et al*., 2009).

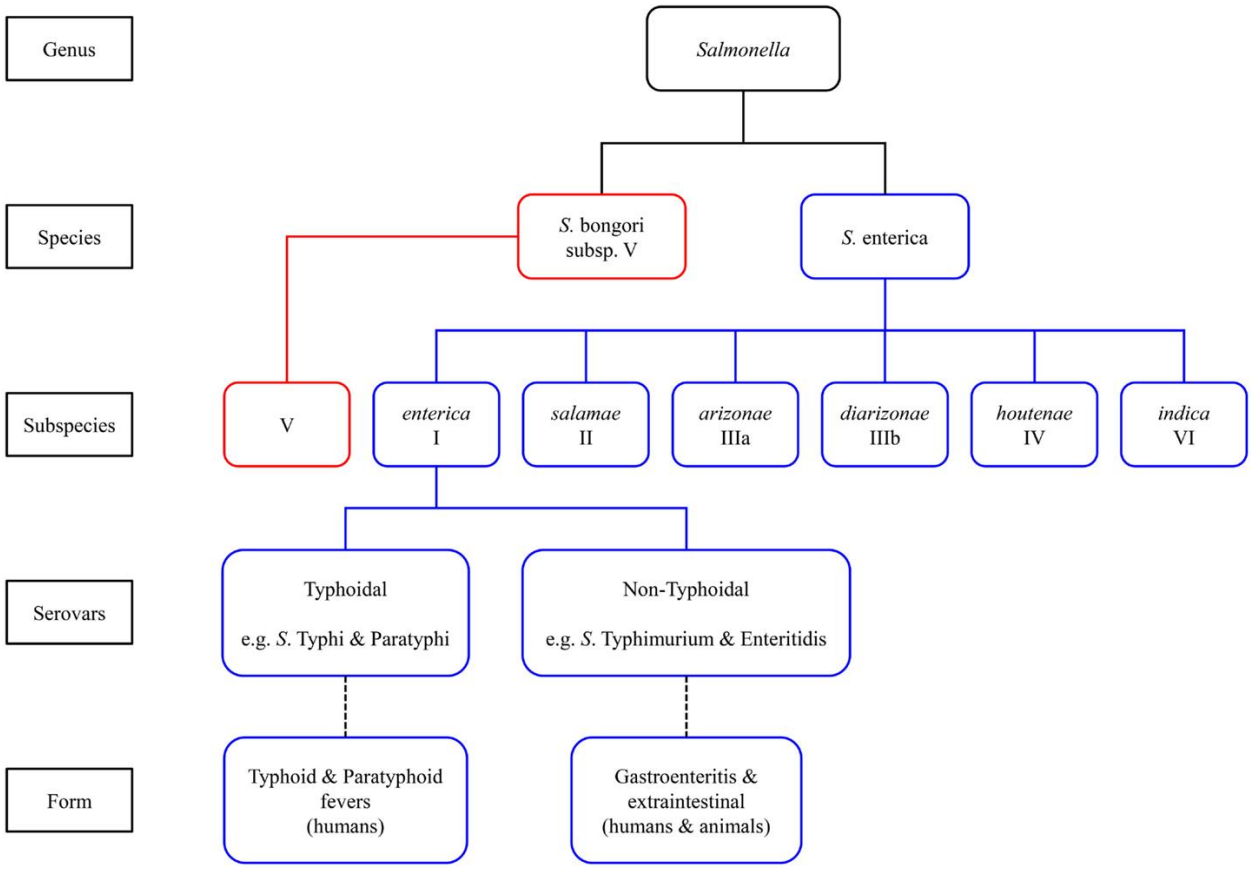

Figure 1-1 Classification of *Salmonella* species and subspecies.

Adapted from Hurley D, McCusker MP, Fanning S, Martins M. (2014). *Salmonella*—host interactions—modulation of the host innate immune system. *Frontiers in Immunology* 5(481):1-11.

Table 1-1 Currently recognized number of *Salmonella* serovars

Adapted from Issenhuth-Jeanjean S, Roggentin P, Mikoleit M, Guibourdenche M, de Pinna E, Nair S, Fields P, Weill FX. (2014). Supplement 2008-2010 (no. 48) to the White—Kauffmann—Le Minor scheme. *Research in Microbiology*, 165 (7): 526-530.

***Salmonella* species and subspecies**	**No. of serovars**
S. enterica	2637
S. enterica spp. enterica	1586
S. enterica spp. salamae	522
S. enterica spp. arizonae	102
S. enterica spp. diarizonae	338
S. enterica spp. houtenae	76
S. enterica spp. indica	13
S. bongori	22
Total (genus *Salmonella*)	**2659**

Salmonella is distributed worldwide and is a leading cause of acute bacterial gastroenteritis in people. Except for serovars Typhi and Paratyphi, domestic and wild animals are the natural reservoirs for all other *Salmonella* serovars. This fact highlights the zoonotic potential of this pathogen. In the U.S., 5 of the 10 most commonly isolated *Salmonella* serovars in humans belong to serogroup C (See Table 3-1). Research into veterinary vaccines to reduce the infection of animals by *Salmonella* serogroup C organisms to reduce the likelihood of human foodborne infection is lacking.

The types of human disease that are caused by *Salmonella* are classified into two major categories: typhoidal and non-typhoidal salmonellosis (Coburn *et al*., 2007). Typhoidal disease (or typhoid fever) is a systemic disease generally caused by *S. enterica* subsp. *enterica* serovar Typhi in humans (Coburn *et al*., 2007). *Salmonella* Paratyphi causes a similar but milder disease called paratyphoid fever (Figure 1-1). Other *Salmonella* serovars cause typhoid-like disease in other animals (for example, Dublin in cattle and Typhimurium in mice) (Costa *et al*., 2012). Clinical signs and symptoms of typhoidal disease in humans include bacteremia, fever, nausea,

anorexia, myalgia and headache (Heymann, 2008). Gastrointestinal symptoms are not a primary feature of typhoidal disease. In contrast, non-typhoidal salmonellosis is primarily an infection of the gastrointestinal tract (enteritis) and is characterized by fever, diarrhea, malaise, nausea, and vomiting (Coburn *et al*., 2007).

Prevalence of Salmonella

Meat vs. Milk

Although poultry and eggs are the most commonly implicated foods in cases of human foodborne salmonellosis (Gould *et al*., 2013), many meats, including beef, are subject to *Salmonella* contamination and represent important sources of *Salmonella* foodborne illnesses (Gould *et al.,* 2013; McEntire *et al.,* 2014; Crowe *et al.,* 2015; Laufer *et al.,* 2015). Among 1,965 *Salmonella* outbreaks reported to the CDC between 1973 and 2011 in which a specific food was identified, five percent were attributed to beef. These outbreaks were responsible for 3,684 illnesses, 318 hospitalizations, and five deaths (Laufer *et al*., 2015). Analyses of outbreak data between 1998 and 2008 identified beef in 10% of *Salmonella* outbreaks (Gould *et al*., 2013) and the fourth most common cause of salmonellosis in the U.S. (McEntire *et al*., 2014). In more recent summaries of outbreak data between 2010 and 2014, contaminated beef was responsible for five outbreaks of *Salmonella* (Crowe *et al*., 2015).

According to the CDC, in 2016 an outbreak of *S.* Montevideo occurred in 9 states and was linked to contaminated pistachios. *S.* Montevideo is a serovar that is commonly isolated from cattle (Table 1-2). The contamination of pistachios could have occurred by spreading *Salmonella* containing cattle manure on fields. Another recent *Salmonella* outbreak linked to cattle occurred in 2016. The CDC investigated the outbreak and concluded that 36 people in 10 states were infected with a multidrug-resistant *S.* Heidelberg. This outbreak was linked to dairy

bull calves, so it is unlikely that the outbreak was due to consuming contaminated meat. The human infections likely were a result of handling sick or dead animals. During the investigation of this outbreak, multiple farms and markets were implicated in the spread of the outbreak strain of *S.* Heidelberg.

The *S.* Montevideo and *S.* Heidelberg outbreaks demonstrate the need for cross-protective vaccines. *S.* Montevideo is a serogroup C bacterium, whereas *S.* Heidelberg is a serogroup B bacterium. A broadly cross-protective vaccine could protect cattle from both of these serogroups, and if cattle are protected the outbreaks in people could possibly avoided.

Milk and other dairy products are less likely to contain *Salmonella* because of commercial pasteurization, reducing the likelihood of infections from these items. A portion of the population consumes products that are not pasteurized; therefore, they have the potential to be infected with contaminated dairy products. For example, an outbreak of *S.* Typhimurium in four states occurred in 2002-2003 due to the consumption of unpasteurized milk products (CDC, 2003). More recently, 8 outbreaks in 5 states occurred between 2013-2015 due to the consumption of raw milk or unpasteurized milk products (CDC FOOD Tool). Several *Salmonella* serotypes were implicated in the outbreaks including Mbandaka, Newport, Typhimurium, and Montevideo. Most milk is tested with commercial test kits, such as an enzyme-linked immunosorbent assay (ELISA), before shipping to a processing facility. Milk that is determined to be positive for *Salmonella* contamination is often destroyed.

Fecal Shedding: Dairy vs. Beef Cattle

Salmonella infected dairy cattle can pose a large risk to public health if they are culled and enter the food supply as beef products. The ground beef produced from these animals can contain peripheral lymph nodes that have been colonized by *Salmonella* (pathogenesis is

discussed in detail below). There have been several cases of human *Salmonella* outbreaks traced back to dairy farms (Holmberg *et al*., 1984; Spika *et al*., 1987) The 1996 USDA survey of dairy cattle, National Animal Health Monitoring System (NAHMS) Dairy '96, reported that 14.9% of culled dairy cows were shedding *Salmonella* and 66.7% of dairy cull markets had at least 1 animal shedding the bacteria. The USDA NAHMS Dairy '96 study also found that 27.5% of U.S. dairy operations and 5.4% of milk cows shed *Salmonella*. The USDA NAHMS Dairy 2007 report showed an increase in the percentage of dairy operations and milk cows that shed *Salmonella* when compared to 1996. In 2007, 39.7% of dairy operations and 13.8% of milk cows shed *Salmonella*. The increase of *Salmonella* shedding by dairy cattle indicates that even with increased awareness and better biosecurity practices, the problem of *Salmonella* infections in cattle is an increasing problem that cannot be curtailed by management practices alone. Vaccination for *Salmonella* with a broadly cross-protective vaccine could help reduce the incidence of *Salmonella* shedding by cattle.

The prevalence of beef cattle shedding *Salmonella* has been investigated several times. Dodd *et al*. (2011b) estimated the prevalence of beef cattle shedding *Salmonella* at feedlot entry in the U.S. to be 64.7% and 72.6% at harvest. These numbers suggest that the prevalence of beef cattle that shed *Salmonella* is much greater than the prevalence of dairy cattle shedding *Salmonella*. While beef cattle can shed a number of *Salmonella* serovars, a few serovars make up most of the recovered isolates. According to Dodd *et al*. (2011b), 86% of recovered isolates can be attributed to just 5 serotypes (Table 1-2). It is estimated that about 1% of feedlot cattle are vaccinated against any *Salmonella* serovar. Currently available commercial vaccines have either shown no, or very slight cross-protection against multiple serovars of *Salmonella*. For example, a *S.* Newport bacterial extract siderophore receptor and porin protein vaccine was evaluated for

efficacy in beef cattle. In short, *Salmonella* use siderophores and porins to transport iron into the bacterial cell, which is critical for bacterial survival. The vaccine is designed to induce production of anti-siderophore receptor and anti-porin protein antibodies by the host immune system. Once the antibodies are bound to the targets, the bacteria are no longer able to transport iron, which is critically important for cell homeostasis, and die (Kingsley *et al*., 1995). Dodd *et al*. (2011a) found no difference between *Salmonella* fecal shedding, mortality, or morbidity among vaccinated and control animals.

Another example is a modified live *S.* Choleraesuis vaccine licensed for use in swine, Enterisol SC-54®, was evaluated for efficacy against *S.* Dublin infection in cattle. The vaccine significantly reduced clinical signs and bacterial shedding (Fox *et al*., 1997), but it was never licensed for use in cattle. An increase in the number of animals vaccinated has the potential to reduce the prevalence of beef calves shedding *Salmonella*, but to convince producers to vaccinate their animals, a cross-protective vaccine must be developed that can protect against the most commonly isolated serotypes.

Table 1-2 Most commonly isolated *Salmonella* serovars from beef cattle.

Adapted from Dodd CC, Renter DG, Shi X, Alam MJ, Nagaraja TG, Sanderson MW. (2011). Prevalence and persistence of *Salmonella* in cohorts of feedlot cattle. *Foodborne Pathogens and Disease*, 8(7): 781-789.

Serotype	Serogroup	No. of isolates	Percent of isolates
Anatum	E	347	28.6%
Montevideo	C_1	261	21.5%
Orion	E	206	17.0%
Kentucky	C_3	123	10.1%
Mbandaka	C_1	110	9.1%
Others	Multiple	167	13.8%
Total	NA	1214	

Peripheral Lymph Node Colonization

How *Salmonella* migrate from the gut to systemic tissues has yet to be completely identified, but there are several theories under investigation. One theory is that bacteremia mediates the movement of *Salmonella* from the gut to the peripheral tissues. Septicemia is a common occurrence in calves dying from salmonellosis as described below. *Salmonella* can be isolated from the blood of calves as early as 2-4 hours post infection (de Jong and Ekdahl, 1965; Pullinger *et al*., 2007). de Jong and Ekdahl (1965) performed esophagectomies on calves to prevent dissemination of an experimental *S.* Typhimurium challenge from the gut to the peripheral lymph nodes (PLNs), but they were still able to isolate the organism from PLNs. This study showed that the dissemination of *Salmonella* does not only involve the alimentary canal, and supports the theory of migration of *Salmonella* through bacteremia.

Another mechanism under investigation is that the lymphatic system mediates the movement of Salmonella bacteria from the gut to the PLNs (Uzzau *et al*., 2000; Paulin *et al*., 2002; Pullinger *et al*., 2007; Brown *et al*., 2015). In general it is believed that the bacteria are simply disseminated once in the lymphatic system.

The final theory is that a route of infection that was not previously thought to be an important factor in bovine *Salmonella* infections may play a much larger role than previously thought. Several recent studies have shown that *Salmonella* may be disseminated to PLNs when a cow is infected transdermally by biting flies or hide abrasions (Edrington *et al*., 2013; Gragg *et al*., 2013; Brown *et al*., 2015). A transdermal infection in the area of the PLNs of interest could result in the dissemination of *Salmonella* to the lymph nodes because of the large number of dendritic cells that would be present in the area. Once *Salmonella* becomes phagocytosed by the dendritic cell, it could be transported to the nearest PLN.

PLN colonization is important because these lymph nodes are likely to be contained in ground beef. Unlike mesenteric lymph nodes, which are easily removed, PLNs are very small, and it is not practical to remove all of the PLNs at slaughter. The first study that showed PLNs as a source of ground beef contamination estimated *Salmonella* prevalence in chuck and flank lymph nodes at 1.6% (Arthur *et al*., 2008). Several other studies have estimated the prevalence of *Salmonella* in PLNs from the same regions as high as 88.2% (Haneklaus *et al*., 2012). Even if the prevalence of *Salmonella* infected PLNs are much lower, a high risk of *Salmonella* outbreaks associated with ground beef is assured, especially if the beef is undercooked.

Several *Salmonella* serotypes have been isolated from PLNs. Gragg *et al*. (2013) found that of the serotypes isolated from subiliac lymph nodes in the flank region of feedlot and cull cattle, 44% were *S.* Montevideo and 24.8% were *S.* Anatum. Currently in the U.S., there are no licensed vaccines targeting either of these serovars. These two serovars belong to different serogroups, suggesting that if vaccination is used as a prevention strategy, a cross-protective vaccine will be necessary.

Bovine Salmonellosis

Clinical cases of bovine salmonellosis are usually observed as a syndrome of septicemia, acute or chronic enteritis or abortion. One or more of these syndromes may occur at the same time in the same animal or herd. Animals may also become asymptomatic latent carriers of *Salmonella*, capable of shedding without clinical signs of disease. Young calves, three months or younger, are most commonly affected, although animals exposed to stressful environments are also at risk to contract the disease. Although salmonellosis in cattle can be caused by many different serovars, *Salmonella* Dublin and *S.* Typhimurium are the most common (Meara, 1973; Radostits *et al.*, 1994). *S.* Cerro is also an emerging serovar of interest in the United States,

having caused a large outbreak of antibiotic resistant cases in Wisconsin in 2015 (Valenzuela *et al*., 2017). The source of infection is usually an environment contaminated by the feces of other infected animals.

Salmonellosis in both calves and adult cattle occurs in most countries in the world and has been shown to be economically important in Europe and North America (Hungerford, 1990; Radostits *et al.,* 1994). The cost associated with bovine salmonellosis is due to the cost of clinical disease including death, diagnosis and treatment of clinical cases, diagnostic laboratory costs, cleaning and disinfection, and the cost of control and prevention. Estimated annual costs for salmonellosis have ranged from billions of dollars in the United States to hundreds to millions of dollars in Canada and millions of pounds in United Kingdom. Analysis of five Salmonella outbreaks due to manufactured food in North America gave direct costs with ranges from $36.4-$62 million. There have been few studies on the cost-benefit ratio of preventing Salmonella infection, but it has been suggested that for every $1 spent on investigation and curtailment of an outbreak, there is a savings of $5 (Wray *et al*., 1991).

Pathogenesis of Salmonellosis

Salmonella bacteria are facultative intracellular organisms and survive in macrophage phagolysosomes where they are protected from antibodies and the effect of complement activation (Radostits *et al*., 1994). This is very important in latent carriers, where stress can induce active shedding of *Salmonella*. The outcome of infection with *Salmonella* depends on three factors: the size of the infective dose, host predisposition, and the level of immunity (Barker *et al*., 1993; Rings, 1985; Williams, 1980).

In bovine salmonellosis, either the intestinal tract is the only organ involved, or organisms may spread beyond the intestinal mucosa and mesenteric lymph nodes resulting in

septicemia or bacteremia and dissemination to the liver, tonsils, spleen, lung, and peripheral lymph nodes. *Salmonella* may also localize in the joints, meninges and placenta (Barker *et al*., 1993; Hall and Jones, 1977; Kennedy *et al*., 1993; Rings, 1985; Segall and Lindberg, 1991; White *et al*., 1981).

For enteric lesions, the pathogenesis of this disease has two stages; first, colonization and invasion of the intestine by the bacteria, which is then followed by increased secretion of fluid and electrolytes (Barker *et al*., 1993; Murray 1986). *Salmonella* must be ingested in sufficient numbers for colonization of the small intestine and colon to occur, normally at least 10^4 organisms (Barker *et al*., 1993; Morse and Duncan, 1974; Segall and Lindberg, 1991; Smith *et al*., 1979; Wray and Sojka, 1977; Wray and Sojka, 1978). Any factors that interfere with and disrupt the normal intestinal flora, such as antibiotic treatment, decreased food and water intake, or stress from transport, may enhance colonization in the small intestine (Clarke *et al*., 1986; Hentges and Maier, 1970; Timoney *et al*., 1988).

The ability of the bacteria to attach and invade enterocytes is essential for the development of enteric salmonellosis (Barker *et al*., 1993). The main site of invasion is the distal ileal mucosa, and high numbers of *Salmonella* can be recovered here shortly after oral infection of calves (Watson *et al*., 1995). Pili enable the bacteria to become attached to the surface of enterocytes, facilitating their entrance into the cells. The bacteria also invade the mucosa through the intercellular junctional complexes (Barker *et al*., 1993). *Salmonella* remain intact in the membrane-bound vacuoles in the enterocyte cytoplasm, multiplying and producing enterotoxin (Barker *et al*., 1993). Enterotoxins will be discussed in more detail below. Most of the bacteria are located in the membrane bound vacuoles within macrophages and neutrophils in the lamina propria, part of the gastrointestinal wall under the epithelium and containing several different

immune cells, by 24 hours post infection (Barker *et al*., 1993; Clarke *et al*., 1986). The *Salmonella* may spread to the regional lymph nodes, where further multiplication takes place.

Salmonella infection can stimulate secretion of increased amounts of fluids and electrolytes. Enterotoxins and the inflammatory response following invasion of the intestinal mucosa causes the release of prostaglandins that activate mucosal adenyl-cyclase, resulting in a net secretion of water, Na^+, HCO_3^-, and Cl^- into the intestinal lumen. (Murray, 1986; Rings, 1985; Turnbull, 1979). Malabsorption and maldigestion develop as a result of cellular infiltration of affected intestine and damage to the villi restricting lymph and blood flow, diminishing the absorptive surface area (Rings, 1985).

Virulence Factors

The pathogenesis of *Salmonella* is dependent on the ability of the bacteria to invade and evade host cells in order to survive and replicate. *Salmonella* produce many virulence factors, which are molecules or sets of molecules that enable the bacteria to replicate and disseminate within a host by evading or subverting host defenses. As discussed below, virulence factors such as virulence plasmids, toxins, fimbriae, and flagella, allow *Salmonella* to colonize and survive in an inhospitable environment. Other virulence factors such as *Salmonella* pathogenicity islands that encode protein secretion systems allow the bacterium to penetrate cells and contribute to bacterial persistence and pathogenicity.

Virulence Plasmids

A feature of serovars that more commonly cause systemic infections is the presence of plasmid encoded *spv* (*Salmonella* plasmid virulence) genes essential to bacterial multiplication within the reticulo-endothelial system of hosts (Rotger and Casadesus, 1999). Virulence plasmids house five genes designated as *spv* RABCD (van Asten and van Dijk, 2005). These *spv*

genes promote enhanced intracellular proliferation in intestinal tissues as well as extra-intestinal sites in the host. The *spv* genes are found in nearly all *Salmonella* isolates that are host-adapted to animals, but only a few broad-host-range serovars (Libby *et al*., 1997).

Toxins

Two forms of toxins aid in bacterial invasion by *Salmonella*: endotoxins and enterotoxins. Endotoxins are cell-associated bacterial toxins. Most commonly, endotoxins are imbedded in the outer membrane of Gram-negative bacteria and are synonymous with lipopolysaccharide (LPS). The toxicity of LPS results from the ability of lipid A to perpetuate immune responses such as the release of tumor necrosis factor (TNF)-α, interleukin-1β, and mammalian inducible nitric oxide synthase (iNOS). The release of the pro-inflammatory mediators is an indication of endotoxins causing harm to the host (Khan *et al*., 1998).

Exotoxins are proteins that modify the host environment and cell functions, such as cytoskeleton dynamics, vesicular trafficking, morphology of host plasma membrane, and immune responses in order to gain entry into targeted cells (Spanò *et al*, 2008; Suárez and Rüssmann, 1998). Exotoxins can be considered either cytotoxins or enterotoxins (van Asten and van Dijk, 2005) and are distributed by a simple release into the cytoplasm of cells under the guidance of a chaperone protein or via protein secretion systems such as type III secretion systems or type IV secretion systems (Spanò *et al*, 2008).

Fimbriae

Fimbriae, also referred to as pili, are significant elements for virulence through the initial bacterial invasion of the intestinal tract by mediating adhesion to the epithelial cells of the intestine (Stevens *et al*., 2009). Fimbriae are located on the outer membrane of *Salmonella* and are 2 to 8 nm wide and 0.5 to 10 μm long (van Asten and van Dijk, 2005). Fimbrins, helically

arranged repeating proteins, are the primary components of fimbriae. *Salmonella* fimbriae adhere the bacteria to the M-cells, which are specialized epithelial cells of the intestinal mucosa that transport antigens from the lumen to other cells of the immune system to initiate an immune response (Edwards *et al*., 2000). There have been 13 fimbrial loci determined for *Salmonella*, one of which is the adhesin, FimH. FimH has been shown to assist in the uptake of bacteria into murine dendritic cells independent of secretion systems (Ibarra and Steeele-Mortimer, 2009). It can be theorized that FimH could be a vaccine candidate that could stimulate an immune response that could prevent *Salmonella* from colonizing dendritic cells and to prevent the spread of *Salmonella* through the lymphatic system.

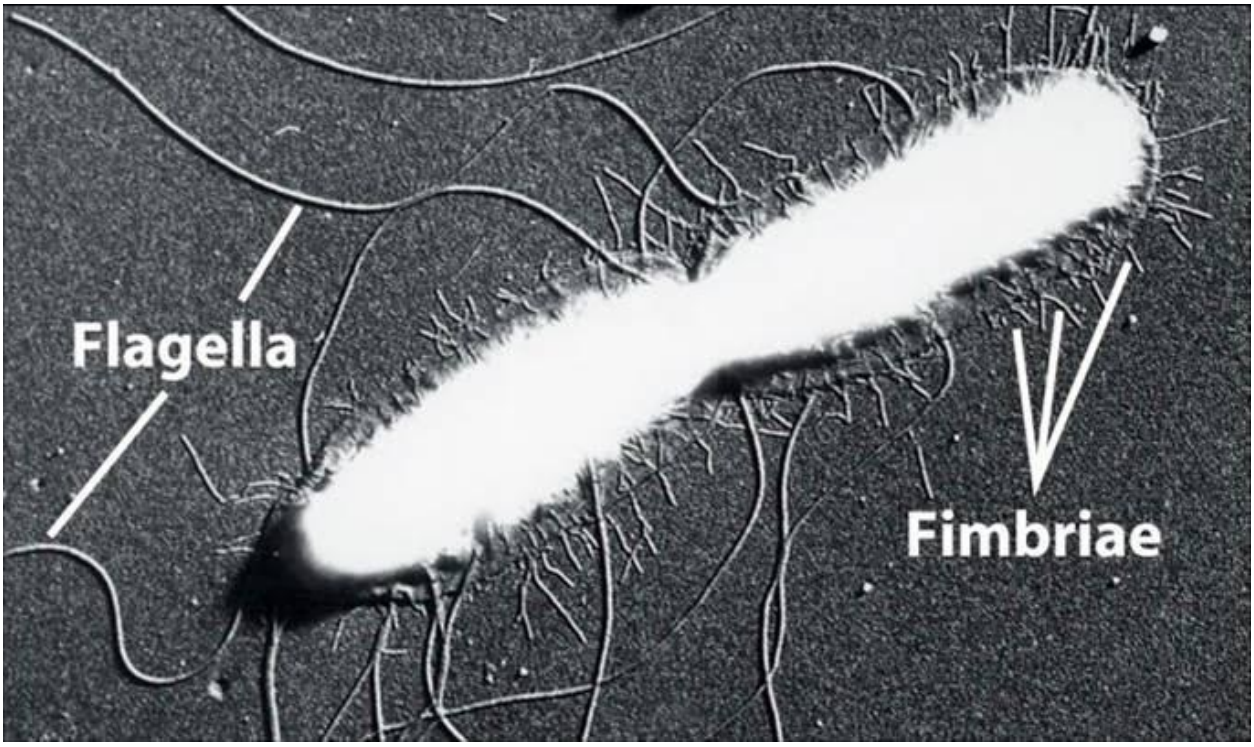

Figure 1-2 A dividing pair of *Salmonella* displaying both its peritrichous flagella and fimbriae.

Adapted from Todar K. (2008) Todar's Online Textbook of Bacteriology Chapter 3: Structure and Function of Prokaryotes. Page 3 of 10. Retrieved from http://textbookofbacteriology.net/kt_toc.html.

Flagella

As a virulence factor, flagella are most important for the initial stage of pathogenicity by allowing *Salmonella* to swim to target cells within the host, facilitating host cell invasion. Flagella are long helical filaments attached to rotary motors embedded in the bacterial membrane and are designed for swimming or swarming (Saini *et al*., 2010). Over 50 genes are required to synthesize the basal body, hook, and a filament of flagella (Uchiya *et al*., 2009). The filaments of flagella are comprised of a protein called flagellin, which is often recognized as pathogen associated molecular patterns (PAMPs) by host's immune system, thereby activating the expression of proinflammatory cytokines (Saini *et al*., 2010; Zipfel *et al*., 2004).

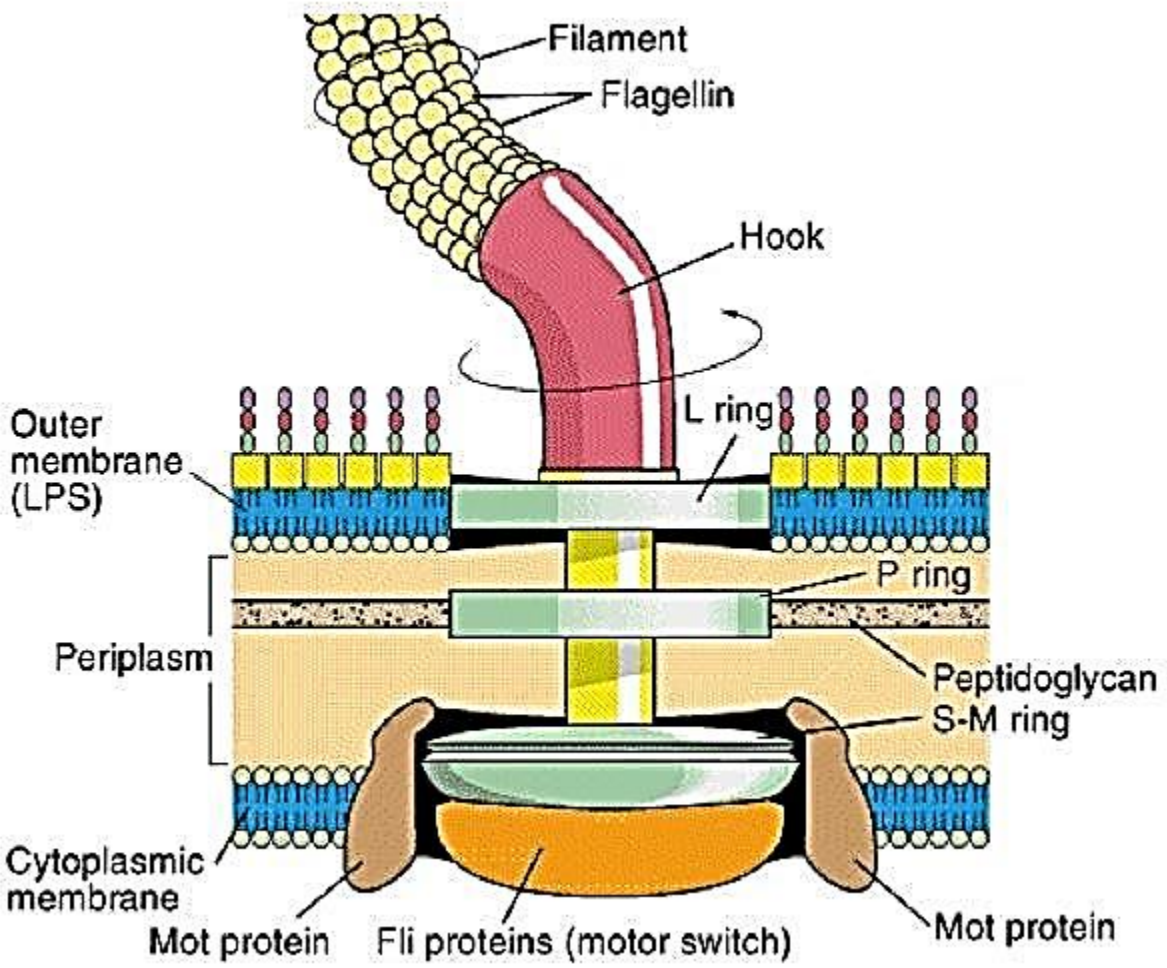

Figure 1-3 The structure of the bacterial flagellum as it resides within the cell wall and membranes.

Adapted from Acharya T. (2013). Bacterial flagella: Structure, importance and examples of flagellated bacteria. *Microbe Online*. http://microbeonline.com/bacterial-flagella-structure-importance-and-examples-of-flagellated-bacteria/

Protein Secretion Systems and Salmonella Pathogenicity Islands

Seven different types of protein secretion systems (types I-VII) have been identified in *Salmonella* (Leung *et al*., 2010). These protein secretion systems allow bacteria to transport

proteins across phospholipid membranes, which may be from the bacteria into the host cell. Type III secretion systems resemble a molecular syringe (Figure 1-4) delivering virulence proteins capable of manipulating host-cell functions such as signal transduction, cytoskeletal rearrangement, membrane trafficking, and cytokine expression. These manipulations allow for bacterial survival and proliferation. Type III secretion system-1 mediates *Salmonella* invasion of epithelial cells, and type III secretion system-2 is essential for macrophage survival (Forest *et al*., 2010; Raffatellu *et al*., 2008). Type VI secretion system plays a role in bacterial infection, intracellular survival, biofilm formation, flagella regulation, quorum sensing, and stress response for *Salmonella* (Leung *et al*., 2010).

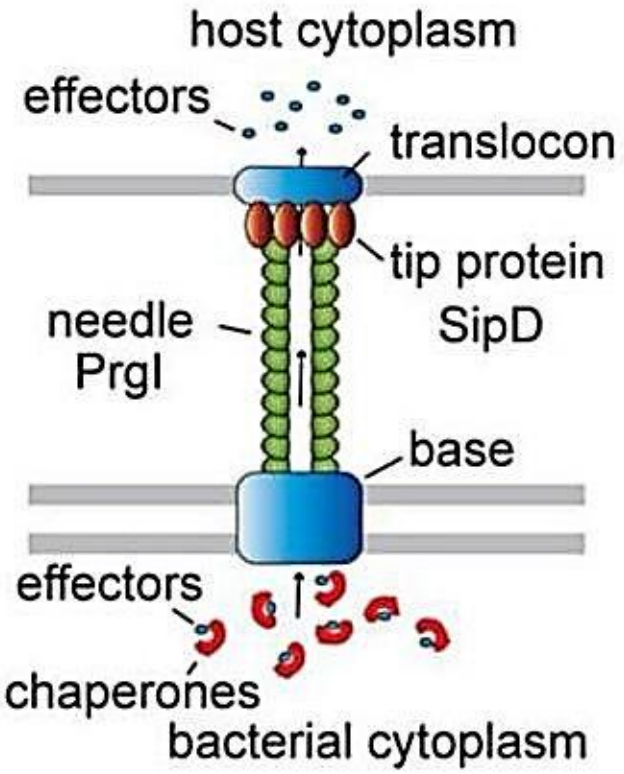

Figure 1-4 Cartoon of the Salmonella type III secretion system.
Adapted from Tang YT, GAO R, Havranek JJ, Groisman EA, Stock AM, Marshall GR. (2012). Inhibition of bacterial virulence: drug-like molecules targeting the *Salmonella enterica* PhoP response regulator. *Chemical Biology & Drug Design*, 79(6): 1007-1017.

S. Typhimurium is capable of releasing proteins via the type III secretion system that provoke its own uptake into target host cells. Once in the host cell, *S. Typhimurium* releases additional effectors via a secondary type III secretion system to impair phagosome maturation, generate *Salmonella*-containing vacuoles (SCVs), and prevent phagosome-lysosome fusion (Bischofberger and van der Goot, 2008; Ohl and Miller, 2001). Type III secretion system-2 can prevent fusion of NADPH-dependent oxidase with SCVs which inhibits the production of reactive oxygen species inside the vacuole. Type III secretion systems are highly conserved, but

the effector proteins released are variable resulting in distinct virulence patterns (Ohl and Miller, 2001).

Type III secretions systems are regulated through *Salmonella* pathogenicity islands, which are clusters of virulence genes located either on the bacterial chromosome or in virulence plasmids (Figure 1-5). There have been 12 *Salmonella* pathogenicity islands identified (Lahiri *et al*., 2010). *Salmonella* pathogenicity island-1 regulates type III secretion system-1 and is essential to the initiation of bacterial infection and invasion of the intestinal tract. *Salmonella* pathogenicity island-2 regulates type III secretion system-2 and is crucial for bacterial survival within host macrophages (Hansen-Wester and Hensel, 2001; Lahiri *et al*., 2010; Suárez and Rüssmann, 1998). *Salmonella* pathogenicity island-2 operons are regulated by a two-component regulatory system, SsrA/SsrB (Forest *et al*., 2010).

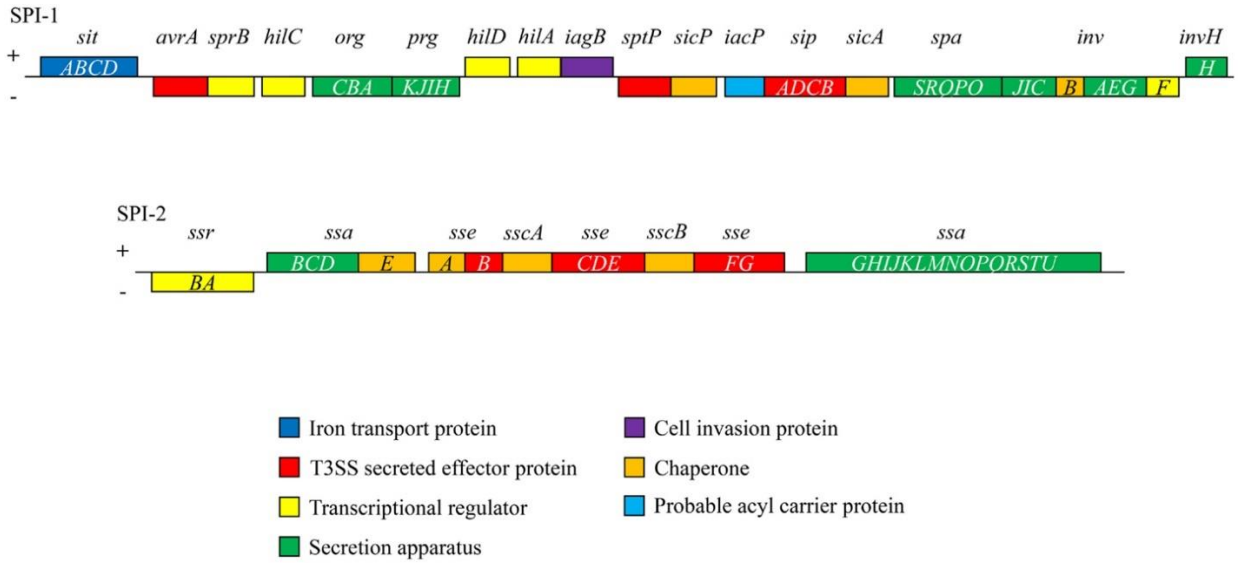

Figure 1-5 Schematic illustration of the genes of *Salmonella* pathogenicity island-1 and *Salmonella* pathogenicity island-2 indicating their functional categories.

In *Salmonella*, salmonella pathogenicity island-1 and salmonella pathogenicity island-2 encode a range of effector proteins, secretion apparatus, and transcriptional regulators in addition to type III secretion system-1 and type III secretion system-2. Adapted from Hurley D, McCusker MP, Fanning S, Martins M. (2014). *Salmonella*—host interactions—modulation of the host innate immune system. *Frontiers in Immunology*, 5(481): 1-11.

Two-Component Regulatory Systems

Two-component regulatory systems allow bacteria to interact with their environment by sensing and responding to changes outside of the bacterial cell (Stock *et al*., 2000). Two-component regulatory systems are comprised of a histidine protein kinase and a response regulator. Changes in the external environment activate the histidine protein kinase, which in turn phosphorylates the response regulator. This phosphorylation results in the activation of an effector domain that produces a response for the change in the environmental condition (Stock *et al*., 2000). These two-component regulatory systems allow *Salmonella* to respond effectively to harsh host environments including nitrogen and phosphate limitation, sugar transport, and

osmolarity, by regulating the effector proteins (Miller *et al*., 1989). One important two-component regulatory system is the PhoQ/PhoP signal transduction system (Figure 1-6). PhoQ is the sensor histidine kinase, and PhoP is the response regulator. PhoQ senses low extracellular magnesium levels, leading to autophosphorylation at a conserved histidine residue. PhoQ transfers to phosphate group to a conserved aspartate residue on PhoP. Phosphorylation of PhoP mediates activation by causing a conformational change, allowing PhoP to homodimerize. PhoP recognizes *phoP* boxes at its DNA promoters and function as a transcription factor to regulate virulence gene expression (Tang *et al*., 2012).

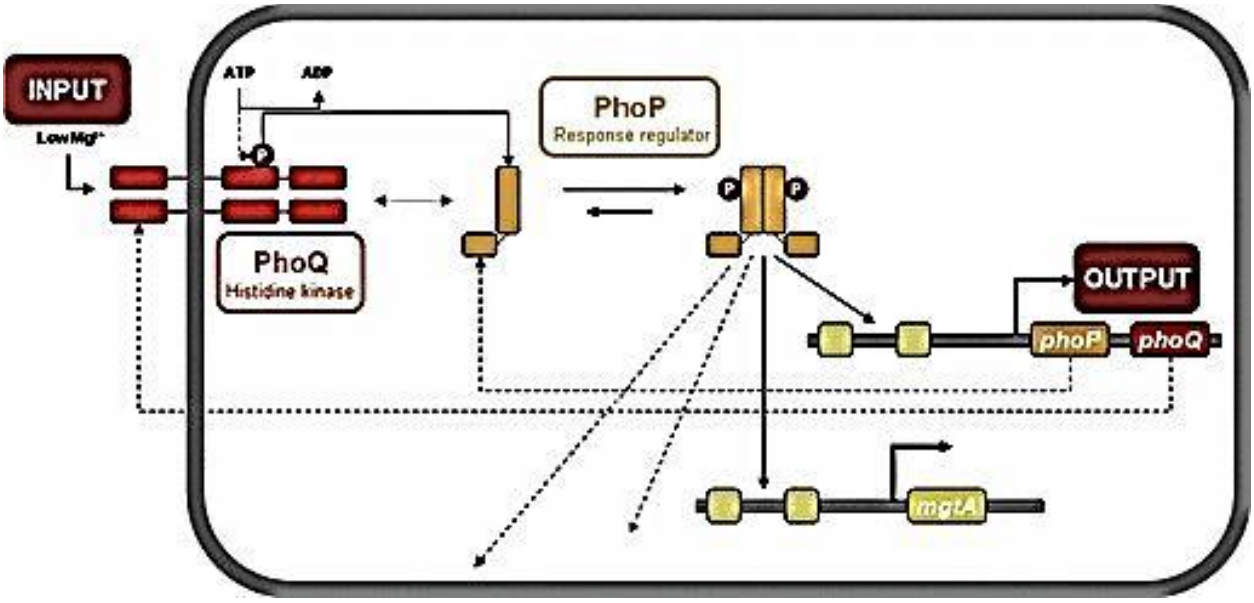

Figure 1-6 Schematic diagram of the Salmonella enterica PhoQ/PhoP two-component signal transduction system.

Adapted from Tang YT, GAO R, Havranek JJ, Groisman EA, Stock AM, Marshall GR. (2012). Inhibition of bacterial virulence: drug-like molecules targeting the *Salmonella enterica* PhoP response regulator. *Chemical Biology & Drug Design*, 79(6): 1007-1017.

Disease symptoms

Calves may develop peracute, acute, or chronic salmonellosis. In addition, it has been found that approximately 5% of apparently healthy calves are asymptomatic carriers of *Salmonella* (McGuirk and Peek, 2003). The peracute disease is usually a septicemic condition that is often fatal. Calves may die suddenly without the onset of symptoms being visualized (Rings, 1985). Some calves develop enteritis and diarrhea in addition to septicemia. (Anderson and Blanchard, 1989; Williams, 1980). Protracted septicemia may result in the development of hepatitis, pneumonia, meningoencephalitis, polyarthritis, and osteomyelitis (Anderson and Blanchard, 1989; Bulgin, 1983; Power and O'Keefe, 1991; Richardson, 1975).

Acute enteric salmonellosis is the most common syndrome of *Salmonella* infection in calves (Anderson and Blanchard, 1989; Rings, 1985; Wray and Sojka, 1977). Calves develop a high fever after an incubation period that is variable between 1-5 days. Other symptoms that may be present are inappetence, lethargy, depression, diarrhea, polypnea, nasal discharge, and coughing (Roy, 1990; Segall and Lindberg, 1991; Smith *et al*., 1979). The feces will smell foul, be a putty-like to watery consistency, and may contain blood, mucus, and sloughed mucosa (Anderson and Blanchard, 1989; Segall and Lindberg, 1991; Smith *et al*., 1979). Calves may show signs of colic, become weaker and dehydrated, and become recumbent. As the disease progresses, body temperature may become subnormal (Wray and Sojka, 1978).

Chronic salmonellosis in calves is characterized by unthriftiness, scruffy hair coats, and stunting of growth (Rings, 1985; Wray and Sojka, 1977). Diarrhea is not always present, but signs of chronic pneumonia and persistent coughing may occur (Hungerford, 1990; Rings, 1985).

Calves that survive any of the three salmonellosis syndromes may develop pneumonia, meningoencephalitis, purulent polyarthritis of the carpal and tarsal joints, and osteomyelitis

resulting in lameness (Gitter *et al*., 1978; Power and O'Keefe, 1991; Rings, 1985; Van den Heever, 1955). Cattle can also become carriers of *Salmonella* and either shed the organism continuously or intermittently. These animals become *Salmonella* reservoirs on farms and can cause an endemic disease state on the farm. Carrier animals can shed up to 10^9 *Salmonella* bacteria in their feces per day (McGuirk and Peek, 2003).

Adult cattle generally contract either acute or subacute enteric salmonellosis. Pregnant animals may abort following infection (Kahrs *et al*., 1972; Williams, 1980; Wray, 1991; Wray and Sojka, 1977). During early stages of the acute enteric disease, animals show fever, depression, inappetence, and low milk yield followed by foul-smelling diarrhea. The feces are usually mucoid with blood clots and shreds of necrotic intestinal mucosa (Carter *et al*., 1983; Richardson, 1975; Stadler and Nesbit, 1990). Affected animals may show signs of colic, congestion, and dehydration (Radostits *et al*., 1994). The acute disease will last about one week with a fatality rate of about 50% (Richardson, 1974; Richardson and Watson, 1971). Animals may take over two months to completely recover (Hughes *et al*., 1971; Williams, 1980).

Animals suffering from subacute enteric salmonellosis present with similar but less severe signs as the acute syndrome (Stadler and Nesbit, 1990; Williams, 1980). Most affected animals recover without treatment (Williams, 1980).

Salmonella Dublin in particular, but other serovars as well, may cause abortion in cows at any stage of pregnancy (Hall and Jones, 1979; Hinton, 1971; Hinton, 1974; Kahrs *et al*., 1972). Abortions may precede the onset of dysentery or follow it in 2-4 weeks. Abortion may also follow pyrexia or may be the only sign of disease (Hall and Jones, 1976; Hinton, 1974; Hughes *et al*., 1971; Richardson, 1971). Decomposition is a common feature in aborted fetuses (Hall and

Jones, 1977). Approximately 70% of cows that abort retain the placenta, but subsequent fertility is not usually affected (Hall and Jones, 1976; Hall and Jones, 1979).

Pathology

Calves affected by peracute septicemic salmonellosis show leukopenia with a left shift, meaning there are an abnormal number of immature neutrophils indicating an acute inflammation. Metabolic acidosis may be present with or without diarrhea or dehydration (Rings, 1985). In calves with enteritis, leukopenia is present early, but leukocytosis and an elevated plasma fibrinogen level develops after three or four days. Dehydration results in elevated packed cell volume and plasma protein levels (Rings, 1985; Smith *et al*., 1979; White *et al*., 1981). Calves suffering from diarrhea have decreased serum sodium and chloride levels, while potassium levels can be either elevated or decreased (Rings, 1985; Wray, 1980). Blood urea levels may rise markedly and be of value in prognostication (Wray, 1980). Plasma bilirubin levels are raised in calves with cholestasis because of interference in bilirubin excretion by bacterial metabolites (White *et al*., 1981). Calves that have died from peracute septicemic salmonellosis have widespread subcutaneous, mucosal, and serosal petechial hemorrhages, generalized congestion, edema and congestion of the lungs, splenomegaly and hepatomegaly (Barker *et al*., 1993; Rings, 1985; White *et al*., 1981).

Calves dying from acute enteric salmonellosis suffer from varying degrees of dehydration and possible mild icterus (White *et al*., 1981; Williams, 1980; Wray and Sojka, 1978). The calves show moderate hepatomegaly, and the liver is bronze or orange-brown. The liver may also have a few to numerous foci of necrosis, 0.5 to 1.0 mm in diameter. There may also be a few petechial hemorrhages disseminated throughout the parenchyma (White *et al*., 1981). The gall bladder may be distended with the wall being edematous, and there may be focal necrosis of its

mucosa or hemorrhages in the wall (Figure 1-9) (White *et al*., 1981). The spleen may be moderately enlarged with necrotic foci in the red pulp, similar to the foci in the liver (Barker *et al*., 1993). Small areas of acute bronchopneumonia, with or without hemorrhages in the lung parenchyma and a fibrinous pleural exudate, may be present (White *et al*., 1981). The kidneys may be swollen with small necrotic foci in the renal cortex (Barker *et al*., 1993; White *et al*., 1981; Williams, 1980). Lesions in the gastrointestinal tract vary in severity and location. *S.* Dublin infections cause multiple erosions and petechiae in the abomasal mucosa (Figure 1-11) (Rings, 1985). In most calves, the jejunum and ileum are affected. Depending on the disease severity, catarrhal, necrotizing, or diphtheritic jejunitis and ileitis may be present (Figure 1-8) (Barker *et al*., 1993; Robinson, 1966; Segall and Lindberg, 1991; Wray and Sojka, 1977). In some animals, the cecum and colon show similar lesions to the small intestine. The contents of the intestine are watery, foul-smelling, and contain fibrin (Figure 1-7), mucus, desquamated mucosa, and digested blood (Wray and Sojka, 1977). The mesenteric lymph nodes are enlarged and edematous with possible petechiae on the interior (Figure 1-10).

Figure 1-7 Fibrin deposits in the small intestine of a calf with salmonellosis.

Adapted from Mohler VL, Izzo MM, House JK. (2009). Salmonella in calves. *Veterinary Clinics of North America: Food Animal Practice*, 35(1): 37-54.

Figure 1-8 Catarrhal hemorrhagic enteritis in a calf with salmonellosis.

Adapted from Mohler VL, Izzo MM, House JK. (2009). Salmonella in calves. *Veterinary Clinics of North America: Food Animal Practice*, 35(1): 37-54.

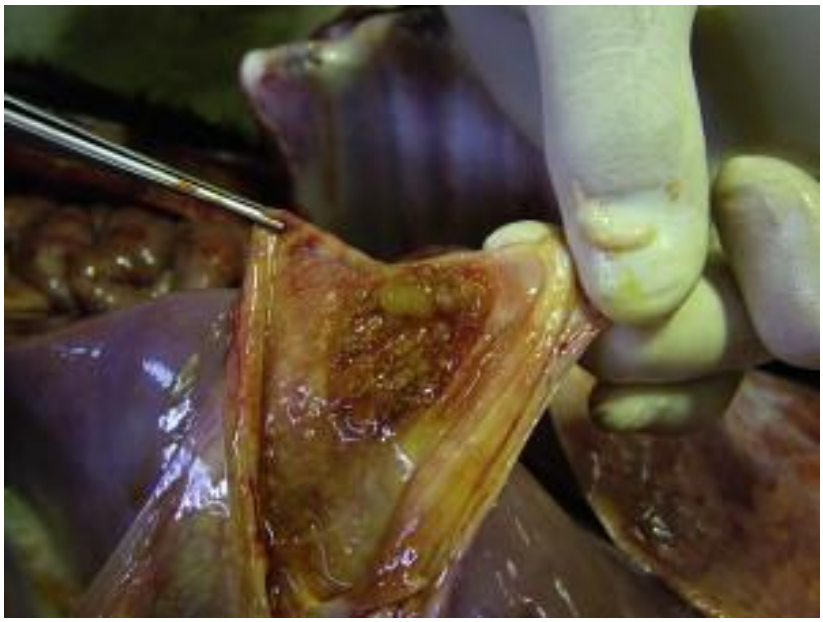

Figure 1-9 Ulcerated bile ducts in the gall bladder.

Adapted from Mohler VL, Izzo MM, House JK. (2009). Salmonella in calves. *Veterinary Clinics of North America: Food Animal Practice*, 35(1): 37-54.

Figure 1-10 Enlarged mesenteric lymph nodes often seen in calves with systemic salmonella infections.

Adapted from Mohler VL, Izzo MM, House JK. (2009). Salmonella in calves. *Veterinary Clinics of North America: Food Animal Practice*, 35(1): 37-54.

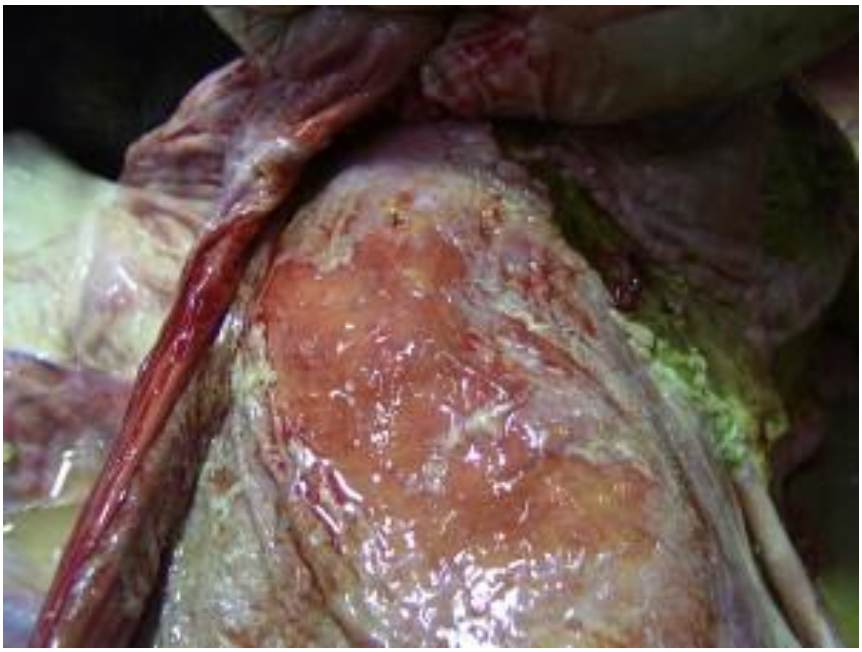

Figure 1-11 Abomasal wall thickening and erosion of the mucosa and submucosa.
Adapted from Mohler VL, Izzo MM, House JK. (2009). Salmonella in calves. *Veterinary Clinics of North America: Food Animal Practice*, 35(1): 37-54.

Calves dying from chronic salmonellosis will show many of the lesions found in the acute disease syndrome; however, lesions of the large intestine will most often be more discrete and necrotic (Radostits, 1994). Signs of localization of infection in a variety of organs and tissues are frequently found (Rings, 1985). Some of these signs are fibrinopurulent synovitis of joints, fibrinopurulent bronchopneumonia and abscessation of the lungs, meningoencephalitis, osteomyelitis of the vertebrae and limbs, and ischemic necrosis of the skin of the ears and tail (O'Connor *et al*., 1972; Power and O'Keefe, 1991; Rings, 1985; Williams, 1980).

Histopathological lesions in the liver comprise small foci of coagulative necrosis infiltrated by a few macrophages and mononuclear cells, referred to as typhoid nodules (Barker *et al*., 1993). Typhoid nodules are randomly scattered in all lobes of the liver, and can be

associated with thrombosis of central veins or blood vessels in portal triads (White *et al*., 1981). Mild to moderate infiltration of mononuclear cells into most portal triads is also present. Vasculitis that is sometimes accompanied by thrombosis of larger blood vessels in the triads, clumps of Gram-negative bacteria in sinusoids, and bile stasis may also be present (White *et al*., 1981). Vascular lesions and necrotic foci similar to those found in the liver are normally present in the spleen, and may also be found in the lungs, lymph nodes, kidneys, and bone marrow (Barker *et al*., 1993).

Lesions in the affected areas of the intestines show necrosis and ulceration of the mucosa, presence of moderate numbers of mononuclear cells and neutrophils in the lamina propria and submucosa, and vasculitis and thrombosis of some blood vessels in the submucosa (White *et al*., 1981). Cellular debris, mucus, and fibrin adhere to the necrotic mucosa or this may be present in the lumen of the affected parts of the intestines. Salmonella bacteria may be observed microscopically as individual bacteria or colonies of organisms in areas of inflammation and necrosis (White *et al*., 1981).

The lesions observed in adult cattle are similar to those observed in calves except that the enteritis is more hemorrhagic and fibrinous or necrotic (Barker *et al*., 1993; Kahrs *et al*., 1972; Stadler and Nesbit, 1990). Mammary infections may lead to chronic-active mastitis and supramammary lymphadenitis (Spier, *et al*., 1991). Cows that abort may show a purulent endometritis and necrotic hepatic foci (Hall and Jones, 1979). The chorioallantois is thickened, the chorionic surface is mottled red and grey, and cotyledons may be covered with a yellow exudate and contain remnants of maternal caruncle (Kennedy and Hibbs, 1993). Mineralization of areas of the placenta may be extensive and some villi contain large numbers of neutrophils (Kennedy and Hibbs, 1993). Many villi will be necrotic and capillaries in the chorionic arcade

will contain prominent bacterial colonies (Hall and Jones, 1979). The fetal liver may have multiple foci of suppuration and neutrophils may occasionally be seen in the bronchi with bacterial colonization of the bronchial epithelium (Kennedy and Hibbs, 1993).

Diagnostic assays

The diagnosis of salmonellosis is based on the isolation of the causative organism, supported by the history, clinical signs, and lesions at necropsy (Anderson and Blanchard, 1989; Stadler and Nesbit, 1990). Specimens submitted to a diagnostic laboratory should be incubated in selective enrichment media, which enhances the chances of isolating the organisms.

To diagnose salmonellosis in live animals, fecal specimens or rectal swabs should be submitted for bacterial isolation (Gitter *et al*., 1978; Palmer *et al*., 1985; Williams, 1980; Wray and Sojka, 1977). If the animal is suffering from severe diarrhea, the volume of feces may dilute the number of organisms to an extent preventing the successful isolation of the organisms from rectal swabs (Rings, 1985; Sojka *et al*., 1974). Salmonella bacteria may be isolated from the blood of septicemic animals (Segall and Lindberg, 1991; Stadler and Nesbit, 1990; White *et al*., 1981; Williams, 1980; Wray and Sojka, 1977). Rectal mucosal biopsy is an easy and safe method to increase the chance of isolating *Salmonella* organisms, because of the adherent and invasive nature of the organism resulting in concentration on the mucosa (Palmer *et al*., 1985).

Fresh liver, spleen, mesenteric lymph nodes, and affected intestinal specimens should be submitted for bacterial isolation (Smith *et al*., 1979). The above samples as well as specimens of the kidneys, brain, and lungs should be fixed in 10% buffered formalin and submitted for histopathological examination. The presence of typical necrotic foci and thrombosis are indicative of salmonellosis (Smith *et al*., 1979).

The diagnosis of carrier animals is more difficult because they shed bacteria intermittently, and it may take a number of fecal specimens before a positive culture is obtained (Wray and Sojka, 1977). Fecal culture and serology testing is usually negative for latent carriers (Wray *et al*., 1989). Since parturition may activate a latent infection, swabs of feces and vaginal discharge should be submitted for culture (Clegg *et al*., 1986; Richardson, 1973). Isolation should be attempted from the gall bladder, ileocecal lymph nodes, tonsils, female genital tract, supramammary lymph nodes and udder if a latent infection is suspected in a dead animal (Lawson *et al*., 1974; Richardson, 1973; Sojka *et al*., 1974; Spier *et al*., 1991; Wood *et al*., 1991).

Salmonella can be isolated from the organs and abomasal contents of aborted fetuses (Hinton, 1971). The placenta and vaginal mucus of cows that abort should also be submitted for bacterial isolation (Richardson, 1974; Williams, 1980).

ELISA, serum agglutination, and complement fixation can be used for the retrospective diagnosis of salmonellosis or the detection of carriers (Carlson *et al*., 1973; Lawson *et al*., 1974 (2), Spier *et al*., 1991; Wray and Sojka, 1977). However, these tests are only reliable when used on a herd basis (Radostits *et al*., 1994; Timoney *et al*., 1988). Antibody responses are much more consistent in animals that have suffered either bacteremia or septicemia (Hinton, 1974; Timoney *et al*., 1988). Positive animals may not have positive serum titers because the serum was collected too early in the infection or the infection was not severe enough to stimulate a detectable humoral immune response (Gitter *et al*., 1978; Radostits *et al*., 1994; Timoney *et al*., 1988). Latently infected cattle will remain serologically negative (Lawson *et al*., 1974).

ELISAs have been developed primarily for the detection of *S.* Dublin or *S.* Typhimurium carriers, but due to cross-reacting O antigens, the assays have poor specificities (Hoorfar *et al*.,

1996; Hoorfar and Wedderkopp, 1995; House *et al*., 1993; Konrad *et al*., 1994). More specific assays have been developed to identify *S.* Dublin carriers using fimbrial antigens or serogroup specific lipopolysaccharide (Hoorfar *et al*., 1996; Konrad *et al*., 1994). ELISAs have also been used to detect antibodies in both milk and blood (Hoorfar and Wedderkopp, 1995; House *et al*., 1993; Konrad *et al*., 1994).

Matrix assisted laser desorption ionization time of flight (MALDI-TOF) and real-time polymerase chain reaction (RT-PCR) have more recently been used in most veterinary diagnostic laboratories when a *Salmonella* infection is suspected. MALDI-TOF is a mass spectrometry assay that can actually detect *Salmonella* and type some of the most commonly isolated serovars from humans and animals (Dieckmann and Malorny, 2011). RT-PCR assays to diagnose *Salmonella* infections are more rapid, cost-effective, and reliable than standard bacteriological methods (Malorny and Hoorfar, 2005). These assays can detect *Salmonella* in very small numbers because they amplify the DNA of the bacteria.

The clinical signs of salmonellosis in calves are very similar to those caused by many other infectious agents that affect the gastrointestinal tract. The most important include bovine coronavirus, rotavirus, and bovine viral diarrhea (BVD) virus I and II, *Escherichia coli*, *Clostridium perfringens*, *Yersinia* spp., *Chlamydia* spp., *Eimeria* spp., and *Cryptosporidium* spp (Radostits *et al*., 1994; Rings, 1985). Over-feeding, incorrect feeding, and feeding of milk-replacers with denatured whey proteins may also cause clinical signs similar to salmonellosis (Radostits *et al*., 1994; Rings, 1985).

The diarrhea associated with enteric salmonellosis in adult cattle can be bloody or dysenteric and should be differentiated from that caused by BVD virus, arsenic poisoning, helminths, and toxic plants (Radostits *et al*., 1994). Abortion caused by *Salmonella* infection

should be differentiated from that induced by other infectious agents such as BVD virus, Rift Valley Fever virus, *Brucella abortus*, and *Leptospira*.

Treatments

Veterinarians disagree about the rationale and wisdom of treating cases of salmonellosis with antimicrobials because of their efficacy and likelihood of producing *Salmonella* carriers (Whitlock, 1984). The choice of drugs to be used ideally would be determined after antibacterial sensitivity of the isolate is determined. Some *Salmonella* isolates may be resistant to multiple antibiotics. However, early treatment based on a likely successful regimen must be used before the results are available. (Osborne *et al*., 1978)

Treatment of salmonellosis in calves is directed at replacing fluid and electrolytes, limiting inflammatory cascades by treatment with non-steroidal inflammatory drugs (NSAIDs), and judicial use of antimicrobials. Parenteral fluid therapy can increase survival rates when administered intravenously (IV). Oral fluids can help animals survive periods of acute dehydration and toxemia. Intravenous hypertonic saline in combination with water or hypotonic sodium-contaminating fluid administered with a stomach tube is a useful treatment. Housing sick animals in a clean, dry, and climate-controlled environment can also improve outcomes.

Chapter 2 - Control and Prevention Strategies

Salmonella infections of cattle can cause great economic losses. More importantly, infected animals can lead to contaminated milk, milk products, and processed beef. It is of vital importance for dairy and beef producers to prevent the introduction of *Salmonella* to their herds and to be able to control the spread of infection if an outbreak occurs. There have been several strategies to control and prevent the infection of calves and adult cattle, including the implementation of certain animal husbandry practices, cleaning and disinfection of animal housing, and vaccination. Each of these strategies should be used as part of a whole control and prevention program.

Animal Husbandry Practices

Producers have control over a few risk factors for their herds based on management practices. The producer should purchase replacement stock from direct sources rather than dealers, quarantine purchased animals for a four-week period, house sick animals in dedicated isolation areas, prevent wild bird access to cattle and feed storage areas as much as possible, and vaccinate their animals. The aforementioned measures are likely to have a great impact on the reduction of salmonellosis (Aarestrup *et al*., 1997; Evans and Davies, 1996).

Treatment and isolation or elimination of animals affected by salmonellosis, as well as disinfection of animal housing areas should be implemented to prevent the buildup of environmental contamination of bacteria (Spier *et al*., 1991). Efforts should be made to detect carrier animals and those animals should be culled from the herd (Spier *et al*., 1991).

Cleaning and Disinfection of Animal Housing

The institution and maintenance of hygiene and good animal husbandry practices is of paramount importance to controlling and preventing *Salmonella* infection. Feces should be removed from housing pens to slurry pits on a regular schedule. Survival of *Salmonella* in slurry is dependent on temperature, oxygen, and pH. Storing slurry in high temperatures, aerobic conditions, and at low pH will decrease the survival of *Salmonella*. According to the New York State Cattle Health Assurance Program (NYSCHAP), spreading slurry on flat land to dry and to be exposed to UV radiation from the sun will also decrease the amount of *Salmonella* that can survive. Feed storage areas should be kept rodent free, separate milk pails that are washed and disinfected after each use should be used for each calf, suitable housing should be provided to prevent overcrowding (Gitter *et al*., 1978). All-in all-out policies with thorough cleaning and disinfection between batches of calves have been effective (Hinton *et al*., 1983).

Many disinfectants are effective against *Salmonella*, but external environments and fomites should be steam cleaned then disinfected with phenolic-, chlorine- or iodine- based disinfectants (Hess *et al*., 1970; Kennedy and Hibbs, 1993; Sojka *et al*., 1974). Five percent formalin can be sprayed several times on earthen floors (Meara, 1973). Carcasses of infected animals should be incinerated. Slurry should be used on crops (that are not root crops), but not on pastures intended for grazing (Radostits *et al*., 1994).

Vaccination

Salmonella infections of calves usually reflect a variety of animal management and environmental factors that result in compromised host immunity and increased pathogen exposure. “Although the implementation of sound nutritional and environmental programs that maintain and/or promote broad-based host immunity contributes to livestock health

and productivity, reliable implementation of these management programs is a formidable challenge with numerous variables to consider, such as weather, mechanical failures, variable feedstuff availability and quality, and labor compliance issues" (Mohler *et al*., 2008).

Vaccination is one of the best forms of prophylaxis against infection by *Salmonella*, but it is often insufficient to be effective on its own (Clegg *et al*., 1986). The effectiveness of *Salmonella* vaccines are influenced by the variety of *Salmonella* serovars that each animal is exposed to as well as the length of time between vaccination and exposure (Mohler *et al.*, 2011). Animals that are exposed to *Salmonella* shortly after vaccination may not have developed an immune response, and the animal may not be protected. Most commercial vaccines do not protect against heterologous challenges, so if an animal has not been vaccinated against the serovar of *Salmonella* it is exposed to, the animal may become infected. It is this type of situation that necessitates the need for the development of broadly cross-protective bovine *Salmonella* vaccines.

The other control and prevention strategies previously discussed also need to be implemented. Immunity to infection by *Salmonella* is thought to require both humoral and cellular immune responses. Humoral immune responses are those that involve antibodies against the pathogen while cellular immune responses are those responses that are cell-mediated, such as phagocyte activation, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines.

Vaccines are generally grouped into three categories: killed or inactivated, live attenuated, and subunit. Live attenuated vaccines are considered to provide more complete and longer lasting immunity to pathogens because they mimic a natural infection without causing clinical disease and confer both humoral and cellular immunity. Killed or inactivated vaccines

generally only confer humoral immunity, and therefore, considered less effective than live attenuated vaccines.

There are very few commercially available bovine *Salmonella* vaccines available in the United States (Table 2-1). Antimicrobial drugs should not be administered within the withdrawal period for the drug prior to or two weeks after the administration of a live attenuated *Salmonella* vaccine because the vaccine strain bacteria will need to be able to colonize the gut mucosa to be effective.

Table 2-1 Current commercially available bovine *Salmonella* vaccines in the U.S.

United States Department of Agriculture Current Veterinary Biologics Catalog. July 5, 2017. https://www.aphis.usda.gov/animal_health/vet_biologics/publications/CurrentProdCodeBook.pdf Accessed on 23July2017.

Name of Vaccine	Company	Type of Vaccine	*Salmonella* Serovars Protected Against
SRP® Salmonella[1]	Zoetis	Subunit	Newport
Super Poly-Bac®	Texas Vet Lab, Inc.	Killed	Typhimurium
Salmonella Dublin-Typhimurium bacterin	Colorado Serum Company	Killed	Dublin, Typhimurium
SDT Guard™	Boehringer Ingelheim Vetmedica	Killed	Dublin, Typhimurium
Entervene®-D	Boehringer Ingelheim Vetmedica	Live Attenuated	Dublin
Salmo Guard®	Agri Labs	Killed	Dublin, Typhimurium
Endovac®	IMMVAC, Inc.	Killed	Typhimurium[2]

1) Vaccine granted only a conditional license. Efficacy tests are in progress.
2) Claims broad cross-protection against Gram-negative bacteria. Only licensed for protection against *S.* Typhimurium.

Killed Salmonella vaccines

Almost all of the commercially available bovine *Salmonella* vaccines in the U.S., 5 of 7 vaccines, are killed vaccines (Table 2-1). Killed *Salmonella* bacterins are normally administered twice with two to four weeks between doses. Bacterins are usually ineffective in calves under six

weeks of age. Bacterins are sometimes administered to pregnant cows to provide calves with colostral immunity, which can last for approximately six weeks (Radostits *et al*., 1994).

Of the 5 commercially available killed vaccines in the U.S., 4 claim protection against just 1 or 2 serovars. Endovac®, licensed by IMMVAC, Inc., claims broad cross-protection against 99% of Gram-negative bacteria. Endovac® is a *Salmonella* Typhimurium bacterin-toxoid that uses an antigen without the outer O-side chains. This exposes the cell wall of the naked core bacterin. Since the cell wall antigens of Gram-negative bacteria are conserved, IMMVAC, Inc. is confident in claiming the broad cross-protection. Endovac® has been tested for its efficacy to prevent clinical coliform mastitis, which has been proven. The vaccine may also protect against endotoxemia in calves caused by multiple *Salmonella* serovars, but it has not been proven to be efficacious against enteric salmonellosis (McClure *et al*., 1994).

An inactivated commercial *Salmonella* Typhimurium vaccine is effective against *S.* Typhimurium challenge (Steinbach and Meier, 1990), but the vaccine does not confer protection against heterologous challenges. This vaccine was combined with other killed antigens to create another vaccine that is commercially available in the U.S.

Immunization of dams 7 and 2 weeks prior to parturition with formalin-killed *S.* Typhimurium is safe and effective for preventing illness in calves (Jones *et al*., 1988) when challenged with a homologous *S.* Typhimurium. This type of vaccination strategy gives a short-lived passive immunity transferred to the calf via colostrum (Cortese, 2009).

Live Attenuated Salmonella vaccines

Live, attenuated *Salmonella* vaccines have been assessed in cattle. These vaccines are considered to induce better protection than killed vaccines due to their ability to stimulate both cellular and humoral immune responses (Villarreal *et al*., 1998). The most widely tested strains

are auxotrophic strains. These strains can only grow when they are provided with a specific substance. Live, attenuated vaccines are generated mainly through targeted gene deletion of genes shown to be necessary for the pathogen in the host. Aromatic amino acid (*aro*) and purine (*pur*) auxotrophs are attenuated and stimulate protective immunity (Stocker, 1988). Purine and histidine auxotrophs of *S.* Typhimurium and *S.* Dublin have been administered orally in cattle in Germany for over 20 years with no reversion to virulence detected (Meyer *et al*., 1992; Rabsch *et al*., 2001), but they confer protection only against homologous challenges.

Live vaccines should carry defined and multiple attenuating mutations and genetic markers to distinguish them from wild-type strains and to prevent the bacteria from reverting to a form that can cause disease. For example, a double mutated *S.* Typhimurium strain at gene locations *aroA* and *aroD* has been shown to protect calves from virulent *S.* Typhimurium (Jones *et al*., 1991; Villarreal *et al*., 1998).

The only commercially available modified live bovine *Salmonella* vaccine in the U.S. is Entervene®-D, licensed by Boehringer Ingelheim Vetmedica. The vaccine strain in Entervene®-D is a *S.* Dublin with a gene deletion in the *aroA* gene. Entervene®-D is administered as a 2 mL subcutaneous dose with a booster dose administered two weeks later. The efficacy of this vaccine was examined in 2011 by Habing *et al*. The research group concluded that administered as the label described, the vaccine reduces clinical disease in calves challenged with only a homologous *S.* Dublin, but the vaccine does not confer protection when administered off label as an oral vaccination.

A modified live *S.* Choleraesuis vaccine administered by intramuscular injection reduces the fecal shedding of serogroup C1 organisms by both cows and calves (House *et al*., 2001). The vaccine confers protection against heterologous challenges, but not challenges that don’t belong

to the same serogroup as *S.* Choleraesuis. The limited cross-protection of this vaccine could lead to a more broadly cross-protective vaccine. This vaccine has been licensed for use in swine, but not for use in cattle.

Subunit Salmonella vaccines

Subunit vaccines are composed of fractions of pathogens that are purified or are generated using recombinant DNA technology. Subunit vaccines are designed to isolate antigens that elicit immune responses without adverse reactions or immunosuppression (Liljeqvist and Ståhl, 1999). Only 1 subunit *Salmonella* vaccine is commercially available for use in cattle in the U.S. Zoetis has licensed SRP® Salmonella, which is composed of highly purified extracts of *S.* Newport proteins known as siderophore receptors and porins (SRP). In short, SRP allow for the transfer of iron into the cell, a critical requirement for bacterial survival. In one field trial, SRP Salmonella had no effect on fecal prevalence of *Salmonella* or health and performance of the animals (Dodd *et al*., 2011a)

The few commercially available bovine *Salmonella* vaccines available in the U.S. lack the ability to confer cross-protection against heterologous challenges to vaccinated animals. Most of the vaccines are killed bacterins, which have a limited efficacy even against homologous challenges. None of the killed bacterins confer cross-protection. One live attenuated vaccine is currently available in the U.S., but the vaccine only protects against homologous challenge and needs to be administered more than once. Only one subunit *Salmonella* bovine vaccine is available in the U.S. The lone subunit vaccine did not show any effect in one field trial. The subunit vaccine is also only conditionally licensed. Since all of the available vaccines have significant limitations, researchers should focus on the development of a broadly cross-protective *Salmonella* bovine vaccine.

Chapter 3 - Future Challenges and Research Opportunities

Although Salmonella vaccines are commercially available for cattle (as discussed in Chapter 2), more research needs to be performed due to several reasons. The limited options of commercially available bovine *Salmonella* vaccines, there are only 7, means that producers are all giving the same vaccines. The current vaccines all have severe limitations. None of the vaccines are cross-protective against challenge with serovars belonging to different serogroups. The one available subunit vaccine, SRP® Salmonella, was found not to be effective in one field trial. Researchers need to focus on developing more effective and broadly cross-protective *Salmonella* vaccines.

Limitations of currently available vaccines

Salmonella Typhimurium and *S.* Enteritidis are heavily researched. Most licensed veterinary *Salmonella* vaccines are targeted to elicit protection against these two serovars. Since so much research effort is dedicated to *S.* Typhimurium and *S.* Enteritidis, they belong to serogroups B and D respectively, serogroup C serovars are not targeted to the extent that they should be. Of the 10 serovars most commonly isolated from humans in the United States, 5 belong to serogroup C (See table 3-1). Of the serovars most commonly isolated from cattle 3 belong to serogroup C. *S.* Dublin (serogroup D), *S.* Cerro (serogroup K), *S.* Anatum (serogroup E) are also serovars of interest when discussing *Salmonella* in cattle. All three of these serovars have been isolated from human clinical *Salmonella* infections. *S.* Anatum is rarely implicated in human disease, but is the most commonly isolated serovar from cattle (Table 1-1).

Of the 10 most commonly isolated *Salmonella* serovars in humans, 7 of the serovars are also isolated in cattle (CDC, 2013). This fact highlights the zoonotic potential of *Salmonella*. As discussed in Chapter 1, ground beef and milk and dairy products have been implicated in many

outbreaks of human salmonellosis. To reduce this risk, a broadly cross-protective vaccine capable of preventing enteric disease and reducing fecal shedding of *Salmonella* bacteria in cattle needs to be developed. The current vaccines lack the capability to protect cattle against *Salmonella* serovars that belong to the same serogroup, and they also do not protect against challenge with serovars from different serogroups than the vaccine strain.

Table 3-1 Most commonly isolated *Salmonella* serovars from humans in the United States.

Information obtained from the Centers for Disease Control National Enteric Disease Surveillance: *Salmonella* Annual Report, 2013

https://www.cdc.gov/nationalsurveillance/pdfs/salmonella-annual-report-2013-508c.pdf

Rank	Serotype	Serogroup
1	Enteritidis	D
2	Typhimurium	B
3	Newport	C_2
4	Javiana	D
5	Heidelberg	B
6	Infantis	C_1
7	Saintpaul	B
8	Muenchen	C_2
9	Montevideo	C_1
10	Braenderup	C_1

Salmonella can infect many organs in cattle, and there is a potential for it to contaminate beef products. Processed beef may become contaminated by contact with hide that has *Salmonella* on the surface or by the addition of infected PLNs in ground beef (Olafson *et al*., 2016). Vaccines for cattle have been developed to either prevent enteric disease in cattle or to reduce carriage in the PLNs, reducing the likelihood of bacterial contamination of beef products.

Interestingly, the peripheral lymph nodes can become infected without an animal suffering from enteric disease. The mechanism of infection in these cases is most likely associated with biting arthropods, especially horn flies (Olafson *et al*., 2016).

Need for cross-protective vaccines

Human foodborne *Salmonella* outbreaks are occurring with unprecedented regularity (CDC, 2012). *Salmonella* control efforts are difficult due to the bacterium's high tolerance to environmental stress, adaptability, multidrug resistance and widespread distribution in animal and environmental reservoirs (Mahan *et al*., 2012). Control efforts are expensive with recent estimates of $14.6 billion annually in the U.S. (Gilliss *et al*., 2011; USDA, 2011; Scharff, 2010). The intensification of livestock production and the emergence of strains with greater capacities to cause human and animal diseases will likely increase the health and economic costs related to *Salmonella* infections. According to the Foodborne Diseases Active Surveillance Network (FoodNet), *Salmonella* causes more foodborne disease than any other food-borne pathogen in the U. S. (Gilliss *et al*., 2011; Scallan *et al*., 2011). Many *Salmonella* serovars infect both humans and animals.

The diversity of *Salmonella* found on farms and feedlots, as well as the potential for strains that are more capable of producing disease in animals and humans, necessitates the use of prophylactic strategies that are effective against several serovars. For many years, antibiotics have been given therapeutically and prophylactically, but this strategy is becoming limited under the Veterinary Feed Directive (VFD). The VFD was written due to the emergence of multidrug-resistant bacteria, including *Salmonella*, which present a risk to human health (Brichta-Harhay *et al*., 2011; Hur *et al*., 2012). Vaccination is one of the best forms of prophylaxis against infection

by *Salmonella*, but commercially available vaccines confer protection limited to a specific strain or closely related set of strains.

Salmonella of several different serotypes from several serogroups are capable of infecting cattle. For example, *S.* Heidelberg and *S.* Typhimurium are both serogroup B organisms, *S.* Newport is found in serogroup C, *S.* Dublin is in serogroup D, and *S.* Cerro is in serogroup K, all of which infect cattle. All of these serovars are also capable of infecting humans as well. According to the CDC National Enteric Disease Surveillance: *Salmonella* Annual Report, 2013, all of the aforementioned serovars have caused multi-state outbreaks in humans through consumption of contaminated ground beef, milk or other dairy products.

Development of a bovine vaccine that displays broad cross protection against *Salmonella* in multiple serogroups is a difficult proposition, but it is a challenge that many research groups are trying to solve. The variety of virulence factors with varying epitopes between serogroups, and even between serovars of the same serogroup, is a challenge that no researcher has been able to overcome yet. However, there are some vaccines that have the possibility of cross-protection.

Convincing producers to vaccinate their herds for each *Salmonella* serogroup individually, is a near impossibility. Even if vaccines were developed that would protect animals against an entire serogroup, adherence to a vaccination schedule with 3-5 *Salmonella* vaccines is not practical. The best way to convince dairy and beef producers to vaccinate their animals against *Salmonella* is to develop a single vaccine that is, not only economical, but broadly cross-protective against multiple serogroups.

Vaccine challenge model development

Since recent research has determined that *Salmonella* bacteria commonly harbors in PLNs of cattle presented for harvest (Arthur *et al*., 2008; Haneklaus *et al*., 2012; Koohmaraie *et*

al., 2012). A challenge model that is predictable and reproducibly causes infection of PLNs by *Salmonella* would be ideal in order to prove efficacy of vaccines. The main goal of many vaccines is to reduce the carriage of *Salmonella* by the PLNs, since PLNs are frequently included in ground beef. If infected lymph nodes are included in ground beef, there is a possibility of an outbreak in humans, especially if the beef is not completely cooked. Edrington *et al*. (2013) developed a transdermal challenge model for the infection of PLNs by *Salmonella.* In short, the research group used a 10-lancet allergy testing instrument to transdermally inoculate calves with either *Salmonella* Newport or *Salmonella* Montevideo in one of the hind legs. Each calf was also inoculated by the transdermal route with *Salmonella* Senftenberg in the lower abdomen. The PLNs were predictably infected after inoculation by the transdermal route and the serogroups recovered from the PLNs that drain the challenge region matched in all but one instance, suggesting a reproducible regionally specific *Salmonella* infection of PLNs.

Oral *Salmonella* challenge models of calves have been around for many years. The development of oral *Salmonella* challenge models is centered on the induction of enteric disease symptoms that would occur in natural infections by ingestion of fecal material. The challenge dose is determined for each *Salmonella* isolate by testing multiple doses and comparing the severity of disease based on a clinical scoring system. The clinical scoring system will normally include, but is not limited to, the following: appetence, fecal appearance, dehydration, animal activity (lethargy), and rectal temperature. A successful challenge model allows for the development of disease over several days, without killing animals due to endotoxic shock. Dependent on the challenge isolate, *Salmonella* organisms may be isolated from feces, small intestine, lung, liver, spleen, mesenteric lymph nodes, gall bladder, joint fluid, and PLNs.

Targets for candidate vaccines

Many researchers are focusing their efforts on developing *Salmonella* subunit vaccines for cattle. Recent research, primarily associated with many of the *Salmonella* virulence factors discussed in Chapter 1, has revealed new antigen targets for vaccine development. Some of the targets described below are conserved among *Salmonella*, so they will potentially be able to elicit an immune response that will protect against many serovars.

Proteins of the type III secretion system

The type III secretion system is a virulence factor for all *Salmonella*, as discussed in Chapter 1. The type III secretion system is a needle-like structure that deliver virulence proteins to target host cells. Type III secretion system-1 mediates *Salmonella* invasion of epithelial cells, and type III secretion system-2 is essential for macrophage survival (Forest *et al*., 2010; Raffatellu *et al*., 2008). The virulence proteins delivered to the host macrophage through the type III secretion system-2 allow for the bacteria to control SCV membrane dynamics, position SCVs within host cells, control immune modulation and cytoskeletal modifications, and affect the motility of infected cells.

The proteins at the tip of the type III secretion system are conserved among *Salmonella* serogroups and serovars, making these proteins good candidates for a cross protective subunit vaccine. Several research groups are working on subunit vaccines focusing on the proteins of the type III secretion system. Based on previous work on the type III secretion system tip protein of *Shigella flexneri*, IpaD, the tip protein of the *Salmonella* Typhimurium type III secretion system, SipD, has been targeted as a potential vaccine candidate (Markham *et al*., 2010). Mice injected with SipD displayed high antibody titers and had increased titers upon booster vaccination. More than one booster vaccination did not show a marked increase in antibody titer. SipD antibody

responses were lower in magnitude than for IpaD, suggesting that this protein may be less immunogenic (Markham *et al*., 2010). Mice were also injected with a complex of the needle proteins, Prgl and SipD. This complex resulted in a lower IgG response than when injected with SipD alone. This result would indicate that a subunit vaccine containing only SipD would be more efficacious than a vaccine containing both Prgl and SipD.

The Markham research group should challenge mice with a heterologous challenge, preferably a serogroup C serovar for reasons explained in Chapter 2. If the vaccine can protect against a heterologous challenge, the research group should conduct a study to evaluate the efficacy of the vaccine in cattle. Based on previous work with a related bacterium, *Shigella flexneri*, the SipD vaccine has the potential to protect calves against enteric disease caused by *Salmonella* infection.

Outer membrane proteins

Outer membrane proteins that are conserved between many serovars of *Salmonella* may be good targets for subunit vaccines. Outer membrane proteins are exposed to the antigen presenting cells of the immune system. This leads to a greater chance of recognition of a pathogen, because the outer membrane proteins are more accessible. Two of the proteins identified as being suitable vaccine candidates are outer membrane protein A (ompA) and phoP-activated gene C (pagC).

Outer membrane protein A has been shown to activate dendritic cells. Lee *et al*. (2012) proved that exposure to a purified ompA from *S.* Typhimurium caused "phenotypic and functional maturation of dendritic cells". This research shows that a vaccine made from ompA could be effective in stimulating an adaptive immune response.

PhoP, described in Chapter 1 as a portion of a two component regulatory system, is the transcriptional regulator for pagC. When pagC is singly mutated, the virulence of *S.* Typhimurium in mice is affected. PhoP also regulates the transcription of other proteins. Multiple mutations in the PhoP regulated proteins could result in highly attenuated, but still efficacious, live vaccine strains (Miller *et al*., 1993). Since PhoP and pagC are highly conserved among many *Salmonella* serovars, these could be potential antigen targets for a cross-protective vaccine candidate.

A suicide vector has been developed to delete the pagC gene and replace it with other antigen genes within the pagC locus (Miller *et al*., 1993). If pagC is not antigenic enough to elicit a strong immune response, a more immunogenic antigen could be inserted into the pagC locus and will be transcribed when PhoP would normally be regulating the transcription of pagC. This could possibly result in a live attenuated strain of *Salmonella,* which produces an antigen that can confer cross-protection against several *Salmonella* serovars.

Fimbrial proteins

As described in Chapter 1, fimbriae are virulence factors of *Salmonella*. One specific fimbrial protein, CsgF, has been identified as a possible vaccine candidate. CsgF is a protein found in a specific type of fimbriae, curli. Curli are a type of proteinaceous cell surface filament produced by enteric bacteria, such as *Escherichia* and *Salmonella*, that facilitate cell adhesion and invasion, bio-film formation, and environmental persistence (Green *et al*., 2016). The function of curli is as a chaperone molecule to help CsgA assemble into curli (Green *et* al., 2016). If a subunit vaccine is developed against curli, the adhesion to and the invasion of the gut mucosa by all *Salmonella* serovars could potentially be avoided.

Flagellar proteins

The major component of *Salmonella* flagella is fliC. Flagella are also virulence factors of *Salmonella* that allow the bacteria to be motile. A recombinant fliC protein vaccine has been tested for efficacy in chickens. The vaccine showed limited efficacy and further improvement is needed, but the bacterial counts in organs normally affected by *Salmonella* infection was significantly reduced when challenged with a homologous organism (Okamura *et al*., 2012). To create a subunit vaccine capable of cross-protection from flagellar proteins, fliC from several serotypes associated with cattle would need to be included in the vaccine.

Future Research

Future research will need to focus on correlation between antibody response and its ability to provide protective immunity in mice and then in cattle. This protection needs to be evaluated for both transdermal and oral challenge models. As vaccines are developed that can protect mice and/or cattle against a homologous challenge, they should be evaluated for cross-protection against serovars in the same serogroup as well as serovars in other serogroups.

An extensive literature review showed no research of proteomic arrays *Salmonella*-specific immune targets for the serotypes that most commonly infect cattle. A proteomic array was developed and 8 new immune targets identified for *Salmonella* Typhi (Lee *et al*., 2012). A similar research project could be conducted for several *Salmonella* serotypes to identify more antigenic components of the bacteria. To do this, the genome of isolates of *Salmonella* to be used would need to be sequenced and open reading frames chosen based on if they code for single proteins or their homology to other *Salmonella* serovar isolates. As the Lee (2012) research group performed, the proteins would then be expressed and printed onto nitrocellulose

membranes. The arrays could then be probed with serum from infected and noninfected mice and cattle to determine which proteins gave only significant responses to sera from infected mice or calves. Any proteins that are found to give significant responses to sera from infected animals and to multiple serovars could then be evaluated further for the potential to be used in cross-protective vaccines.

As discussed earlier in this report, live attenuated vaccines are widely considered to confer the best and longest lasting immune response. Developing a live attenuated vaccine that can confer cross-protection should be of high importance. While many groups are researching live attenuated vaccines, none have been found that are broadly cross-protective.

Mutations in the *dam* gene of *Salmonella*, preventing expression of the DNA adenine methylase, also result in highly attenuated organisms (Heithoff *et* al., 2015). Vaccination of calves with an attenuated *S.* Typhimurium *dam* strain conferred protection against calves via both adaptive immunity and competitive exclusion mechanisms (Dueger *et al*., 2003). Immunization of young calves with a similar strain resulted in reduced clinical disease, reduction of fecal shedding, and reduction of lymph node colonization by heterologous *S.* Dublin and *S.* Newport challenge compared to non-vaccinated animals (Mohler *et al*., 2006, 2008). This vaccine strain needs to be evaluated for efficacy against more heterologous challenges, especially challenges of *Salmonella* serovars contained in Table 1-2. This vaccine is not licensed and is not commercially available.

Cross-protective *Salmonella* vaccine development is an area of interest of several research groups as described above. Each research groups has a different approach to vaccine development. Some research groups are developing subunit vaccines. One limitation of subunit vaccines is that many times the amount of antigen needed to confer protection makes the

production of the vaccine cost prohibitive. Sometimes addition of adjuvants, materials designed to stimulate a stronger immune response, can alleviate this issue, but many times it cannot.

At least one research group has developed a live attenuated vaccine strain of *Salmonella* Typhimurium that can protect against heterologous challenges of one serovar in each of serogroup C and D. Live attenuated vaccines have an inherent advantage over subunit vaccines in that the live vaccine strains will replicate within the host for a time, producing more antigen load than what was in the original vaccine. Live attenuated vaccines also rely on more than one antigen of the vaccine strain. These two factors increase the possibility that a live attenuated vaccine will be not only efficacious, but it will also be economical.

Chapter 4 - Discussion and Conclusions

Salmonella occurs worldwide and is a leading cause of acute bacterial gastroenteritis in people. *Salmonella* infection is the leading cause of foodborne illness in the U.S. Ground beef is one of the major causes of human *Salmonella* infections in the U.S., with milk and other dairy products also contributing to the number of human *Salmonella* infections. While there are many control and prevention options, a comprehensive program should be developed. Vaccination should be part of any *Salmonella* control and prevention program because it is one of the most effective forms of prophylaxis against the development of salmonellosis.

Several serotypes of *Salmonella* can infect cattle and can also be shed by cattle. These serotypes belong to several serogroups. Since serovars are defined by their poly-O (cell wall) and H (flagellar) antigens, and these antigens are also considered virulence factors, vaccines developed for one serovar may not be protective for other serovars or serogroups. A cross-protective *Salmonella* vaccine would solve a problem of vaccinating animals for several *Salmonella* serotypes.

Most beef and dairy producers will not adhere to a vaccination schedule that requires multiple vaccinations for *Salmonella*. According to the USDA NAHMS Dairy 2014 Report, only 2.5% of dairy operations vaccinated cattle against *Salmonella.* The USDA NAHMS Beef 2007-08 Report shows only 0.5% of cow-calf operations vaccinated against *Salmonella*, while the USDA NAHMS Beef 2011 Report shows that only 5.2% of feedlot cattle are vaccinated against *Salmonella*. These facts makes the development of a cross-protective vaccine a priority. Many research groups are working towards the development of just such a vaccine. There are many strategies among the research groups, and the strategy depends on what part of the disease the

vaccine targets. Some vaccines will target peripheral lymph node colonization, since the peripheral lymph nodes have been implicated in contamination of ground beef. Other vaccines will try to prevent the gut mucosa from being colonized so that fecal shedding is reduced and carrier animals are not produced from subclinical infections.

Most vaccines that are being researched to prevent the colonization of the peripheral lymph nodes are subunit vaccines. The most successful subunit vaccines so far are made of proteins of the type III secretion system, one of the many virulence factors of *Salmonella*. The potential for these subunit vaccines to be cross-protective comes from the fact that the type III secretion system needle tip proteins are highly conserved among *Salmonella* serovars.

Live attenuated vaccines are being developed to prevent the colonization of the gut mucosa and to reduce the amount of shedding of *Salmonella* in the feces. Live attenuated vaccines are preferable to subunit vaccines because they generally confer humoral and cellular immunity. Live attenuated vaccines also have the potential to present many antigens to the host, any one of which could be cross-protective to another serovar. Several research groups seem to be close to developing a live attenuated vaccine that confers protection against several *Salmonella* serovars, but so far none are broadly cross-protective.

With continued research, a vaccine that is cross-protective against several serovars and serogroups is obtainable. For now, research should focus on vaccines that protect cattle against serotypes in serogroups E, C_1, and C_3 since serotypes in these serogroups account for most of the isolations of *Salmonella* from cattle. Of the 10 *Salmonella* serovars most commonly isolated from humans (Table 3-1), seven of them are also isolated from cattle. Another serogroup, D, should be targeted for vaccine development because serovars from that group, such as *S.* Dublin, tend to cause a more severe clinical disease in calves.

The development of cross-protective *Salmonella* vaccines will give producers another option to combat a disease for which a large amount of money is spent to control and prevent. These cross-protective vaccines could also increase the productivity of the cattle and protect our food supply.

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