DISSERTATION

MUCOSAL IMMUNIZATION OF MICE WITH A RECOMBINANT *SALMONELLA CHOLERAESUIS* THAT EXPRESSES A MULTIMERIC GONADOTROPIN RELEASING HORMONE FUSION PROTEIN

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ABSTRACT

MUCOSAL IMMUNIZATION OF MICE WITH A RECOMBINANT *SALMONELLA CHOLERAESUIS* THAT EXPRESSES A MULTIMERIC GONADOTROPIN RELEASING HORMONE FUSION PROTEIN

Parenteral vaccines effectively induce protective immunity against a variety of infectious diseases. However, they are impractical as a means to mass vaccinate free-ranging, wild, or feral animals since they are injected, and the animals must therefore be captured. Oral delivery is the most logical and practical method of vaccinating these types of animals. The delivery of oral vaccines, however, presents many challenges. First, an oral vaccine must be environmentally safe, must remain stable over a prolonged time period, and should not cause secondary pathogenicity. In addition, to be effective, an oral vaccine must deliver sufficient immunogen to elicit a strong host immune response; similar to that induced by a parenteral vaccine and sufficient to generate protective levels of antibody against targeted pathogens. Oral vaccines that utilize modified or attenuated live vectors offer a reasonable approach to immunize populations of domestic, wild, and feral animals against infectious pathogens and parasites. Although live vectors are the method of choice for orally vaccinating wildlife, they are inherently "short lived" because exposure to elevated temperatures within the delivery device for extended periods can destabilize them.

Recently, wildlife and feral animal management programs have proposed using vaccines to reduce the transmission of zoonotic pathogens from wildlife to domestic livestock. Brucellosis, for example, can be spread from from North American elk (*cervus elaphus nelson*) and feral swine (*Sus scrofa*), to domestic livestock herds. These animals are routinely vaccinated to help protect them from potential transmission of these diseases. Recently, immunocontraception has been proposed as a non-lethal means for indirectly controlling disease transmission and as a population management tool for specific populations of feral dogs (*Canis familiaris*), swine (*Sus scrofa)*, and horses (*Equus caballus*), as well as a wide range of wildlife species including white-tailed deer (*Odocoileus virginianus*), European badgers (*Meles meles*; Europe), black-tailed prairie dogs (*Cynomys ludovicianus*), and brush-tailed opossums (*Trichosurus vulpecula*; New Zealand).

Various modified *Salmonella* vaccines have been developed to protect against a variety of disease pathogens. Domestic swine can be protected against salmonellosis by administering an oral vaccine using avirulent *Salmonella choleraesuis* strain 54 (SC54). In the first portion of this dissertation I describe the engineering required for the oral delivery of live avirulent recombinant *Salmonella choleraesuis* strain 54 (**rSC54**) that expresses a multimeric gonadotropin-releasing hormone fusion protein (rmGnRH), which stimulates the production of anti-GnRH antibodies when injected intramuscularly into an animal. Using the rSC54 as an oral delivery vector, the rmGnRH protein is expressed within the bacteria and exposed to the mucosal-associated lymphoid tissue (MALT) of outbred CD-1 laboratory mice (*Mus musculus*), inducing an anti-rmGnRH antibody response. These anti-rmGnRH antibodies bind to endogenous circulating GnRH,

immunoneutralizing GnRH and inhibiting normal leutinizing hormone (LH) and follicle stimulating hormone (FSH) activity and downstream reproductive physiology.

The second portion of the study describes a method for thermally protecting live rSC54 in a dry, non-crystalline matrix that is capable of maintaining bacterial immunogenicity at elevated temperatures for prolonged periods of time. By preventing degradation of intracellular protein and ensuring metabolic stability through temporary cessation of cellular processes, the live vector can potentially be incorporated into an oral bait without loss of viability or immuno-stimulatory characteristics. The use of this thermal stabilization process to protect and stabilize live rSC54 provides a novel tool for future development of wildlife and feral animal management programs.

Experiment 1 describes the molecular engineering of the pNS2TrcD-rmGnRH expression plasmid. This plasmid is able to propagate in a wide variety of gram-negative bacteria, thus making it an attractive candidate for use in *Salmonella*. The recombinant multimeric GnRH (rmGnRH) fusion protein encodes for five GnRH amino acid sequences interspersed with four T-cell sequences from various zoonotic disease organisms, making the protein highly foreign and immunogenic to the host immune system. Results from this experiment showed successfully cloned expression plasmid constructs at ~500bp (rmGnRH) and ~3500bp (pNS2TrcD-rmGnRH) observed via agarose gel electrophoresis.

Experiment 2 describes the transformation of pNS2TrcD-rmGnRH into *Salmonella choleraesuis* strain 54 (SC54) and *in vitro* induced protein expression that shows stably-expressed rmGnRH fusion protein.. After the induction, the cells were lysed and the expressed rmGnRH protein was observed via SDS-PAGE and measured for immunoreactivity using immuno-blot analysis. Results showed positive protein

expression after 2 hours of induction and a greater concentration of protein was observed after 4 hours. Immunoblot analysis showed immunoreactivity against purified rabbit anti-rmGnRH antibody. As a control, a second immuno-blot showed immunoreactivity against mouse anti-his antibody to detect the 6X histidine tag connected to the protein and to determine if the protein was completely expressed.

Experiment 3 describes the oral delivery of recombinant rSC54 to CD-1 mice with comparative analyses of serology, sperm motility and concentration, and testicular morphology. Mice were assigned to one of 7 treatment groups including rSC54 oral gavage (n=9), rSC54 nasal lavage (n=9), subcutaneous injection of heat-killed rSC54 (n=9), subcutaneous injection of live rSC54 (n=9), intramuscular injection (IM) of purified rmGnRH protein emulsion positive control (n=9), and oral gavage of non-modified SC54 as a control (n=9) and a no treatment negative control group (n=7). Serum samples were collected monthly for 3 months and analyzed for anti-rmGnRH antibody concentrations via enzyme-linked immunosorbent assay (ELISA). Testicular and epididymal tissue were obtained at necropsy and evaluated for sperm concentration and motility, tissue weight, and histological characteristics. Results from the experiment showed a moderate decrease in sperm concentration and testis size, and an antibody response in mice that were orally gavaged with rSC54 and the positive control group when compared to untreated and control mice. Mice that were treated nasally with rSC54 showed a delayed immune response with antibody titers increasing after a boost was administered. Mice that were injected subcutaneously did not generate a significant antibody response compared to negative control mice.

Experiment 4 describes the development of a drying process capable of stabilizing the live rSC54 bacterium in a solid carbohydrate matrix. The process known as

“foamation” uses both vacuum and temperature manipulations to arrest cellular machinery, thereby inhibiting degradation of live biologicals within the solid matrix. Two different bacterial carbohydrate matrices using sucrose and trehalose were exposed to ambient (25°C) and above-ambient (37°C) temperatures for three weeks with testing of bacterial viability at 1 week intervals. Differential scanning calorimetry (DSC) was used to measure the melting points of the matrices to determine the limits of thermal stability. The results showed that the majority of bacteria that were stabilized using the foamation process were successfully protected against a prolonged exposure to elevated temperatures of 37°C, while DSC results showed that the carbohydrate matrices remained intact when exposed to higher than ambient temperatures.

Currently, the most practical vaccination route for wildlife is through oral delivery. Live, self-replicative attenuated bacteria and viruses offer the most effective means in stimulating a protective immune response originating from oral exposure. Development of vaccines and effective strategies to vaccinate wildlife species is an arduous task and there is an increasing demand for management of feral or free-ranging animals since most have become “pest” species and some serve as reservoirs of infectious disease. Anti-fertility vaccines aimed at controlling animal numbers as a means for controlling the spread of disease has been proposed in a variety of settings, including brucellosis and rabies vaccination programs. Immunocontraception, using anti-fertility vaccines, is developing into a viable management tool for regulation of animal numbers in specific groups of captive, feral or free-ranging, and wild animals.

This research determined that modified, self-replicative, intracellular bacteria, produced a highly immunogenic multimeric GnRH fusion protein, and that the process of foamation could provide for a stable delivery mechanism that protects the immunogen

from proteinaceous degradation. Together these methods may provide a practical means of immunocontraception for overabundant wildlife and domestic animal species.

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Chapter I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Oral vaccination of wildlife represents a potential tool for controlling transmissible diseases that represent threats to human health or livestock production. Wildlife vaccine design and vaccination strategies pose unique challenges for researchers due to various requirements that must be met during vaccine development and application. For example, whereas parenteral vaccines are very effective in inducing strong immunologic responses in a variety of domestic animals, they are impractical for free-ranging wildlife species. The most logical method for vaccinating wildlife against infectious disease is through oral immunization. Therefore, the main objective of current wildlife vaccine design efforts is to develop oral delivery devices that present high concentrations of potent antigen or vaccine to an animal while maintaining vaccine efficacy for extended periods of time at or above ambient environmental temperatures, prior to the animal consuming bait that contains the vaccine.

Oral vaccines that utilize live, self-replicative modified or attenuated bacteria, like *Salmonella*, elicit strong protective immunologic responses. This form of oral immunization has been highly successful in eliciting immune responses against wildlife infectious diseases such as tuberculosis (Mollenkopf et al., 2001) and plague (Garmory et al., 2003; Morton et al., 2004). These microorganism vectors in these situations have been genetically altered and made avirulent to avoid causing the disease normally

associated with exposure to the pathogen. But the immunologic response observed is a production of antibodies against the invading organism, virus or bacteria, or other foreign molecules or genetic information that the vector may carry.

For a robust systemic immune response to produce protective immunity, sufficient concentrations of immune-stimulating agent, or immunogen, must be delivered to the mucosa-associated lymphoid tissue (MALT). Various methods of adapting an immunogen to suit the delivery mechanism, to extend the biological activity, and to inhibit its degradation have been investigated including freeze-drying (Follmann et al., 2004) and lipid encapsulation of BCG (Aldwell et al., 2003b). In both cases, refrigeration is necessary to maintain viability during long-term storage, and there is general loss of vaccine efficacy if exposure to inhospitable environmental conditions persist for long periods. An ideal wildlife vaccine would need to be protected from environmental exposure without loss of immunologic efficacy. Therefore, the delivery vehicle must provide both environmental protection and stable biological payload containment.

Novel methods of biological stabilization are being developed that can thermally protect live microorganisms, proteins, and viruses from high-temperature degradation. Similar to preservation techniques using lyophilization, a thermostabilization process, known as 'foamation' dries live biologicals in a non-crystalline matrix above freezing temperatures and arrests their molecular machinery. This cessation of molecular movement prevents normal degradation, and biologicals are protected from degrading at elevated temperatures (Bronshtein, 1997). In addition, this process results in a solid material, facilitating incorporation into oral bait matrices. Foamation techniques may provide added stabilization and extended viability of an oral wildlife vaccine through protection against elevated temperatures or other extreme environmental conditions.

Prior to the introduction of broad-scale rabies disease management campaigns, culling was used extensively to control the transmission of the disease among wild animals (Bradley et al., 1999). Although these campaigns reduced the number of diseased or potentially diseased animals in the wild, they did not reduce overall disease transmission (Cross et al., 2007). For that reason, investigation into alternative means for controlling disease transmission has been of great interest among wildlife biologists and public health officials. Recently, wildlife researchers and management programs have proposed using anti-fertility agents alone, or in tandem with culling operations, to assist in controlling infectious disease transmission and population densities of both feral and wild animals (World Health Organization, 2009).

Anti-fertility vaccines are successful in reducing the fecundity of targeted feral and wildlife species, and they represent a potentially powerful population management tool. These vaccines have been tested in many feral and wild animal models, including feral cats (Levy et al., 2004), dogs (Massei et al., 2010), pigs (Miller et al., 2006), and horses (Killian et al., 2006a), as well as wild white-tailed deer (Gionfriddo et al., 2006, 2008, 2009; Killian et al., 2006b), black-tailed prairie dogs (Yoder and Miller, 2010), brush-tailed opossums (Eckery and Thomson, 2007), and Tammar wallabies (Hinds et al., 2009). In contrast to vaccines that protect against infectious disease, anti-fertility vaccines induce an auto-immune response to "self" antigens and induce the production of antibodies against endogenous molecules that are naturally present in the treated animal (Hardy and Braid, 2007). Several parenteral anti-fertility vaccines comprised of "self" immunogens are now commercially available (e.g., ImproVac™ Commonwealth Science Laboratories, Australia and Vaxstate™ Arthur Webster, Australia).

As a tool for wildlife management, an oral anti-fertility vaccine is needed that elicits the same immunologic protective response generated by parenteral anti-fertility vaccine products. Using broad-scale fertility control has not yet been demonstrated as an effective method for controlling disease transmission or reducing population densities in wildlife. Preliminary modeling studies, however, have shown reasonable outcomes when adding this approach to traditional wildlife management activities (Yoder et al., 2008). Using knowledge from various areas of research including molecular biology, wildlife disease biology, oral vaccine development, and formulation chemistry, new tools to control reproduction and pathogen transmission can be accomplished.

1.2 Oral Immunization in Wildlife

In recent years, wildlife species, including coyotes (Stull and Mengak, 2009), black-tailed prairie dogs (Nash et al., 2007), and white-tailed deer (Miller et al., 2008), have become pest species, due to conflicts with humans as human populations have moved into previously wild areas. These human-wildlife conflicts are associated with wildlife habitat loss and the removal of natural predators, which cause populations of other animals to increase to unstable levels (Lloyd-Smith et al., 2005). In addition, these overabundant animal populations act as reservoirs for pathogens that can infect humans and domestic livestock (Boyle, 1994).

In some cases, the attempts to reduce or eliminate a disease from these wildlife reservoirs is made through lethal control of the vector population (Cross et al., 2007). For example, large-scale culling operations over vast geographical regions, where rabies was endemic, were not found to be cost-effective, and they offered only a temporary reduction in the transmission of the disease. By the late 1970s and early 1980s, large-scale rabies oral vaccination programs had been implemented and have been highly effective in

controlling the spread of rabies among meso-carnivore populations in the endemic areas along the eastern seaboard of the United States (Rupprecht et al., 2006).

A considerable amount of research led to the development of an oral rabies vaccine (ORV), targeted for raccoons, to help reduce the spread of rabies. The federal campaign established to inoculate wild animals with this vaccine has been highly methodological and successful. The vaccine utilizes a potent live recombinant orthopox vaccinia virus that carries the genetic sequence for the rabies glycoprotein (G). It is enclosed in a biodegradable plastic sachet that is embedded in an edible fish-meal polymer block. When the animal bites the sachet releasing its contents into the animal's mouth, the vaccinia virus primarily invades the oral-pharyngeal mucosal area and generates high levels of Th1 protective antibodies that immunize the animal against rabies.

The success of the ORV program has encouraged additional oral vaccine research and the development of practical strategies for controlling the transmission of infectious diseases in wildlife. The attenuated *Mycobacterium bovis* strain BCG (bacilli Calmette-Guerin) was first developed as an oral vaccine in humans (Gheorghiu et al., 1996), but has been adapted to protect Virginia opossums (*Didelphis virginiana*) and other wildlife against tuberculosis (Aldwell et al., 2003a). The ability of the BCG vaccine to reduce transmission of the pathogen and the severity of the disease in Virginia opossums was demonstrated when animals were previously vaccinated and then experimentally challenged with aerosolized *M. bovis*. In addition, *Brucella abortus* strain 51 (RB51), an APHIS-licensed parenteral vaccine to control brucellosis in domestic cattle, has also been used in Yellowstone bison (Olsen et al., 2009). Oral vaccination and protection against brucellosis has also been demonstrated in domestic pigs (Edmonds et al., 2001),

laboratory mice (Pasquali et al., 2003), captive Richardson's ground squirrels (*Spermophilus richardsonii*) (Nol et al., 2009), black bears (Olsen et al., 2004), American pronghorn (Elzer et al., 2002), and coyotes (Kreeger et al., 2002).

Oral delivery is generally accepted as the most practical method for vaccination of free-ranging wildlife species. Vaccines that were not previously used for wildlife species are becoming more widely used to protect against disease and inhibit further transmission of the pathogens to humans and other animals including domestic species.

1.3 Salmonella Vaccines

Live, avirulent or attenuated bacteria offer great potential for developing oral delivery vaccines targeting the mucosal surfaces (Kotton and Hohmann, 2004; Curtiss III, 2002). Attenuated strains of *Shigella, Salmonella spp*., *Yersinia enterocolitica*, and *Listeria monocytogenes* have been shown to stimulate immunological inductive sites at mucosal surfaces, and are therefore attractive candidates for vaccine development (Schoen et al., 2004). *Salmonella spp*. are intracellular, self-replicating, enteroinvasive microorganisms that penetrate and colonize the intestinal mucosa after oral administration (Ogra et al., 2001). *Salmonella* are given selective pathogen entry into specific inductive sites, activating effector T- and B-lymphocytes (Clark et al., 2001) and leading to an immunologic protective state. Because *Salmonella* are invasive and self-replicating, they are an ideal candidate for a modified live-vector wildlife vaccine. Specifically, *Salmonella enterica* target the epithelia of the intestine and the underlying gut-associated lymphoid tissue (GALT). The bacteria traverse the intestinal epithelial barrier and proliferate in the lymphoreticular organs causing a sustained immunologic response against *Salmonella* (Curtiss III et al., 1993). These bacteria are one of the most extensively studied bacterial species utilized for genetic modification and heterologous

protein expression. In addition, a plethora of foreign antigens derived from bacterial, fungal, viral, and parasitic organisms have been expressed within *Salmonella* vectors. However, most research has focused on the development of vaccines to protect humans against infectious diseases such as tuberculosis (Wang et al., 2009), tetanus (Fairweather et al., 1990), pertussis (Dalla Pozza et al., 1998), and hepatitis C (Liao et al., 2007), not wildlife diseases.

Recently, techniques for manipulating genetics have opened up the possibility of introducing potentially protective antigens from other bacterial species or even from viruses and eukaryotic parasites into *Salmonella*. Bacterial vaccines rely heavily on genetic attenuation, which renders a live vector non-virulent but allows the vector to remain viable. This genetic attenuation can be achieved by introducing genetically defined, non-reverting mutations into specific genes in the bacterial chromosome. These bacteria will possess a genetic mutation that makes them incapable of surviving *in vivo* (Hoiseth and Stocker, 1981; Dougan et al., 1987). Therefore, attenuated auxotrophic bacteria are environmentally safe and offer the most realistic approach for developing oral wildlife vaccines. These bacteria require a metabolic supplement not found in natural settings, and therefore bacterial growth and protein expression can be controlled. *Salmonella typhimurium* aroA mutants have been developed that express K88 fimbrial antigen, which is the adhesion complex responsible for the attachment of enterotoxigenic *Escherichia coli* (*E. coli*) (Clements and El-Morshidy, 1984) and LTB, the B subunit of the heat labile entertoxin of *E. coli* (Stevenson et al., 1985). These proteins elicit a strong immune response because they are secreted on the cell surface. Most potentially protective antigens from viruses and parasites are not surface-active proteins like K88 or LTB, and therefore, the antigens are expressed as fusion proteins that accumulate as

inclusion bodies in the bacteria. Although the location of protein expression is different, substantial immune response is generated against intracellular antigens as well (Hess et al., 2000; Kaufmann and Hess, 1999).

An alternative method for attenuating virulent bacteria is by repeated passage through neutrophils, which causes the bacteria to lose their virulence plasmid. This method of attenuation was utilized to develop *Salmonella cholereaesuis* strain 54 (Cheville et al., 1996), which is a gram-negative serovar of *Salomonella enterica*, registered by the United States Department of Agriculture (USDA) as an oral vaccine for protection against salmonellosis in domestic swine (Letellier et al., 2001). The avirulent bacteria are highly immunogenic when delivered orally through drinking water to domestic swine (Kramer, 1995) and protective against *Salmonella dublin* infection in calves (Fox et al., 1997; Hermesch et al., 2008; House et al, 2001).

Immunocontraceptive vaccines achieve long-term efficacy through reliance on the same long-lasting memory B-cell response that is induced by vaccination against infectious diseases with immunologic memory. Considerable research has utilized modified bacteria to deliver anti-GnRH antigens with the goal of developing long-term oral vaccines against prostate cancer in humans (Talwar et al., 2004). In addition, multiple recombinant GnRH fusion proteins have been developed (Gupta et al., 2004; Ladd et al., 1994; Raina et al., 2004; Ulker et al., 2009; Xu et al., 2006; Zhang et al., 1999) and tested in feral and wildlife species including domestic pigs (Miller et al., 2006) and feral dogs (Bender et al., 2009). Recently, a GnRH fusion protein was designed consisting of a multi-copy sequence of mammalian GnRH interspersed with T-cell epitope sequences from a variety of foreign immunogens. T-cell sequences cause the GnRH fusion protein to be recognized as foreign protein, which enhances a strong T cell

response and a subsequent antigen-specific B-cell response is induced against the attached GnRH sequences (Gupta et al., 2004; Sad et al., 1992). Bird et al (1996) showed that a recombinant *S. typhimurium* that expressed the fox LDH-C4 sperm antigen induced a mucosal immune response against the LDH-C4 protein. Similarly, *S. typhimurium* that expressed the human sperm antigen SP10 induced antibody production in mice (Srinivasan et al., 1995). Furthermore, oral immunization of balb/C mice with modified *Salmonella* that expressed murine ZP3, one of the three main glycoproteins associated with zona pellucida, induced significant anti-ZP3 IgG and serum and IgA antibodies (Zhang et al., 1997).

Successful oral immunization can be achieved using attenuated *Salmonella* strains because they are capable of inducing both antibody and cell-mediated antigen-specific immune responses (Russmann et al., 1998). Wildlife species, whose populations sometimes serve as potential reservoirs for infectious disease, have been vaccinated orally with modified or attenuated bacteria that express disease determinants and these show promise as potential vaccine candidates. The use of modified live bacteria to elicit protective anti-fertility immunity in wildlife has yet to be tested on a practical level, although this approach successfully immunized foxes against sperm antigens in a controlled study (De Jersey et al., 1999). Further investigations of this method of oral immunization are needed to develop wildlife vaccines.

1.4 Molecular Techniques

Recombinant technology using host organisms, such as bacteria, to express foreign antigens has made it possible to develop potential recombinant vaccines against infectious diseases and immunocontraceptive vaccines. In addition, recombinant technology can be exploited to produce large quantities of antigen or recombinant protein

from bacteria, for other purposes. Bacterial protein expression depends upon the transgene, or the gene-of-interest that is to be translated into a protein, being surrounded by a collection of signals or genetic sequences which provide instructions for the transcription and translation of the transgene by the bacteria. Using molecules of circular DNA, known as plasmids that are found in bacteria, but are separate from the bacterial chromosome, and adding to the plasmid replication factors such as a promoter sequence, a ribosome binding site, a and terminator sequence. The result is an expression plasmid capable of being transcribed and translated normally by bacteria (Weaver, 2002). Adding the transgene sequence to an expression plasmid and transforming it into bacteria can result in successfully transcribed and translated transgene proteins that can be collected in large volumes from the bacteria, making this process an attractive, cost-efficient method for producing high amounts of recombinant protein (Lodish et al., 1999).

To accomplish this, the plasmid containing the transgene and replication factors is first transformed or inserted into a naïve competent bacterium, which has been exposed to cold temperatures and heavy metal ions like calcium and magnesium causing the cell membrane to become perforated. Plasmids are introduced at this time and are easily taken up by the competent bacteria (Weaver, 2002).

Expression plasmids, which contain unique transgene and replication sequences, are species specific. That is, metabolic mechanisms and DNA replication molecules that operate in *E.coli* do not necessarily function in *Salmonella*. However, some replication factors and plasmids are broad host range sequences and can be used in several bacterial species including *Salmonella* and *Brucella* (Seleem et al., 2008). Various genetic sequences can be added to the transgene sequence or placed upstream within the reading frame of the expression plasmid to enhance over-all gene expression, such as including a

RNA stem loop, a UP element, and a 6X histidine sequence for optimal purification of the expressed protein (Seleem et al., 2007b). A UP element consists of a (A)-rich and A+T rich upstream nucleotide sequence that provides binding sites for the RNA polymerase during transcription (Estrem et al., 1998), and the insertion of a 6 base-pair (bp) RNA stem loop stabilizes the mRNA strand leading to enhanced protein expression (Estrem et al., 1998; Paulus et al., 2004). The attached histidine sequence tag allows for one-step purification of the recombinant protein by nickel chelate affinity chromatography. The tandem histidine residues have a high affinity to nickel ions attached to the resin within a column. Whole cell lysate is added to the Ni+ column and only those proteins that have an associated histidine sequence when expressed will bind to the Ni+, which can then be eluted and concentrated (Qiagen, 2011).

Depending on the size and complexity of the transgenic protein, total protein collection from a 1 liter flask of bacterial culture can yield 10-150mg of expressed protein (Hengen, 1995; Hochuli, 1988). Bacteria commonly express the transgene as an intracellular protein, and store it as an inactive aggregate known as an inclusion body since the bacteria do not normally use these proteins for any metabolic processes (Fink, 1998). These inclusion bodies can be collected and re-folded under denaturing conditions, which consist of exposing the inclusion bodies to chaotropic agents that disrupt the three dimensional structure of macromolecules such as proteins, DNA, or RNA and allows them to dissolve more easily. These expressed proteins can then be collected using column chromatography and concentrated for possible therapeutic or testing purposes (Villaverde and Mar Carrió, 2003).

Inclusion bodies are intracellular bodies and are not secreted on the surface of the bacterial cell, therefore, a delayed somatic immune response is observed since these

proteins are not initially released but only come in contact with the immune system after the cells have been destroyed by macrophages (Kaufmann and Hess, 1999). This is very important when developing an oral vaccine utilizing bacteria that express a potentially protective antigen. A rapid protective T cell response is generated against secreted bacterial proteins. However, oral vaccines should elicit long lasting immunity, thus a continuous release of antigen, or inclusion body breakdown from delayed bacterial destruction by phagosomes may sustain a protective immune response (Hess et al., 1996; Kaufmann and Hess, 1999).

1.5 Bacterial Activation of Mucosal Induction Sites

Live oral vaccines based on enteric activity can activate nearly every effector arm of the immune system. Because of their biology as enteric organisms, they elicit a strong sectretory IgA (sIgA) antibody response that prevents attachment and invasion from microorganisms. They also stimulate serum IgG antibodies that aid in controlling mucosally and systemically invasive pathogens, elicit a cell-mediated immune response against intracellular bacteria and viruses, as well as stimulate antigen-specific B-cell responses that provide long-term protection against infection from re-exposure (Germain and Marguiles, 1993; Paseti et al., 2011). An effective vaccination causes a broad host immune response against mucosal pathogens. This response consists of a pathogen-induced production of cytokines and chemokines, including IL-1, IL-2, IL-6, TNF-alpha, IFN-gamma, IL-12, and IL-15, by epithelial cells and local macrophages (Curtiss III, 2002; Foss and Murtough, 1999; Walker, 2004). This cell signaling activity is a cascade of immunologic cross-talk providing long-term immunization against an antigen, and initiates the process of dendritic cell maturation, Th1/Th2 polarization, and effector cell chemotaxis (Bland, 1998; Galan, 2001).

Oral vaccines are highly dependent on the delivery of live, viable biologicals and the presentation and exposure of sufficient quantities of potent immunogen to inductive sites to elicit a protective immunologic response. In mice, the mucosa-associated lymphoid tissue (MALT) consists of the nasopharynx-associated lymphoid tissue (NALT) and the gastrointestinal-associated lymphoid tissue (GALT) (Bland and Bailey, 1998). These two regions make up the primary inductive sites for a mucosal immune response (Chadwick et al., 2009). These specialized regions of follicle-associated epithelium (FAE) contain various immune cells, including dendritic antigen-presenting cells (APC) that extend cellular arms into the lumen and sample a variety of foreign antigens. In addition, specialized blunted epithelial cells, known as M-cells, have been found in both the NALT and GALT regions (Asanuma et al., 1997; Csencsits et al., 1999; Wu et al., 1996). M-cells transport foreign material from the lumen at their apical membrane to the follicle at their basolateral membrane. In transit through the cell, the material is phagocytosed by macrophages and processed by APCs for antigen presentation to the lymphatic system (Gebert et al., 2004). Foreign bacterial antigens are presented as MHC-class II molecules to both antigen-specific B cells and T cells to elicit a Th1 immune response (Burton et al., 1994; Germain and Marguiles, 1993). Primed B cells proliferate in the gut and other mucosal tissues where clonal expansion and differentiation into plasma cells occurs, and the cells then migrate to the mesenteric lymph nodes and enter the general circulation via the thoracic duct (Cardenas and Clements, 1992). Because of the self-replicative nature of invasive pathogens, they continuously stimulate the immune system, thereby generating a protective immunologic response and long-term protection (Fooks, 2000).

Attenuated bacteria may offer the most practical method for orally immunizing wildlife because they 1) stimulate a strong protective immune response, 2) are easily modified to provide environmental safety, and 3) do not revert to a virulent state. In addition, various foreign antigens have been expressed from *Salmonella* and are therefore an attractive mechanism for delivering antigens associated with infectious disease-causing pathogens as well as "self" proteins. Vaccines that elicit an immune response to "self" reproductive proteins like zona pellucida (ZP) and gonadatropin releasing hormone (GnRH) have been developed for potential application in the management of feral and wild animals.

1.6 Immunocontraception Concepts and Tools

Improving meat quality from food-producing animals, reducing disease transmission from wild to domestic animals, and managing specific populations of feral or wildlife species are all logical goals of immune- or chemical-contraceptive research. Costs of surgically castrating meat-producing animals have encouraged interest in developing non-surgical methods of sterilization (D'Occhio et al., 2000) and several contraceptive agents have been developed, including conjugated subunit vaccines (Bradley et al., 1999) and early ovarian senescence compounds (Mauldin and Miller, 2007).

Immunocontraception can be used to reduce disease transmission to domestic livestock from wildlife and to control reproduction in wildlife (Boyle, 1994). Whereas most current vaccine development is aimed at protecting humans from infectious diseases; anti-fertility vaccines directed towards wildlife are being developed to induce immune responses against endogenous molecules, peptides, and antigens. The most widely tested products include vaccines against mammalian ZP proteins, gonadatropin

releasing hormone (GnRH), and sperm antigens. These parenteral vaccines require either subcutaneous or intramuscular injection and cause the immune system to generate antibodies against the desired endogenous "self" proteins when creating an immunocontraceptive effect.

The ZP vaccines are most commonly derived from porcine zona pellucida proteins. Injection of the purified ZP proteins, the host is induced to generate antibodies against its own ZP glycoproteins (Hasegawa et al., 1992; Jones et al., 1992; Kirkpatrick et al., 1991, 1992). Due to the evolutionarily semi-conserved nature of zona pellucida proteins within mammalian phylogenetic lineages, porcine zona pellucida produces an immune response in many different mammalian species, and the antibodies bind to the "self" zona pellucida within the animal and block sperm binding, penetration and fertilization (Kirkpatrick and Turner, 2002). This method of immunocontraception has been tested with the most success in feral horses (Turner et al., 2002).

Gonadatropin releasing hormone (GnRH) is a major reproductive hormone produced in the hypothalamic neurons and transported via axonal flow to their terminal ends, which synapse on circulatory vessels of the primary capillary plexus within the median eminence (Thompson Jr., 2000). GnRH is released from the hypothalamous in picogram (pg) concentrations and diffuses into the blood-stream, traveling through the hypophysial portal system to the sinusoidal capillary plexus within the andenohypophysis. GnRH then diffuses into the anterior pituitary, binds to gonadatropins there causing these cells to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH exert their effects "downstream" on gonadal tissues which are responsible for sexual development, estrous cycling, and oocyte maturation and release in females; and spermatogenesis in males (Thompson, 2000). Vaccines against

GnRH induce antibodies that form an anti-GnRH complex that either inhibits the hormone's natural diffusion through capillary walls or blocks the receptor binding site on the GnRH molecule. In either scenario, immuno-neutralization of endogenous GnRH occurs, and the downstream reproductive hormonal and subsequent physiological activities are inhibited (Bradley et al., 1999; Jewgenow et al., 2006).

Several vaccines have been tested that cause this neutralization effect. One is GonaCon™ immunocontraceptive vaccine, which is registered with the Environmental Protection Agency (EPA) in the United States. This vaccine has been used to treat adult, female white-tailed deer. GonaCon™ induces antibodies against endogenous gonadatropin releasing hormone. The vaccine consists of a synthetic ten-amino-acid GnRH sequence chemically conjugated to a large eight-million-molecular-weight hemocyanin (a blue protein) derived from the salt water abalone species *Concholepas conchelepas* (Miller et al., 2008). Large keyhole limpet hemocyanin and albumin molecules have also been utilized to generate a strong immune response when conjugated to "self" proteins, including GnRH (Goubau et al., 1989). The size and/or extreme foreignness of the marine invertebrate hemocyanin proteins provide an enhanced immune response from mammalian immune systems. Using these vaccines, very little antibody must be generated to produce immunoneutralization effects against endogenous GnRH as the GnRH-antibody complexes are too large to diffuse into the anterior pituitary and affect the gonadotropic cells there, thus the normal hormonal cycles are broken.

One non-lethal method for reducing overabundant animal populations and potentially controlling the spread of disease from these wild animals to domestic animals is through immunocontraception. As previously mentioned, modified bacteria that induce a protective immune response have been developed to provide practical delivery

mechanisms for vaccinating wildlife. Various studies have shown that these bacteria can be modified to express contraceptive antigens. However, additional research is needed to improve the efficacy, safety, and the ease of delivering these oral immunocontraceptive vaccines to wildlife.

1.7 Stabilization of Biologicals for Wildlife Vaccine Delivery

Freeze-drying, or lyophilization, has been used extensively to store biologicals in a dry state. This process has a number of drawbacks, however; since freezing and subsequent equilibration at moderately low temperatures can damage labile biologicals, the process takes several days, and the lyophilized materials often must still be stored at refrigerated temperatures to ensure stability. In 1956, D.I. Annear demonstrated that viscous solutions and biological liquids containing sugars or amino acids could be dried by foaming during vacuum application and used this process to preserve several types of bacteria in a dry state. Later it was reported that various biological macromolecules could be effectively stabilized by "foam drying" (Bronshtein, 1997). Bronshtein showed that this process allowed biologicals to be stabilized at temperatures higher than ambient with minimal loss of activity during drying and subsequent storage. This foaming technique has been used to stabilize various organisms, including bacteria and viruses as well as various antigenic protein compounds at room temperature or at higher temperatures without significant loss of activity (Alcock et al., 2010; Annear, 1956; Pisal et al., 2006).

Foamation is the transformation of a liquid into a glass, which is a highly immobile, noncrystalline, amorphous, dry, solid state. The ability of certain sugars to form glass matrices when dried can result in anhydrobiosis, whereby cells, cellular machinery, proteins, and viruses are essentially frozen in the glass, allowing for long-term survival of organisms under conditions of extreme dryness. The glass matrix arrests

protein motion, resulting in dramatic conformational stabilization, thereby inhibiting protein denaturation and degradation (Bronshtein, 2003). Preserving an antigenic organism under anhydrobiotic conditions permits the organism to remain intact and antigenic for long time periods at temperatures from 25 to 45°C. Recently, scientists at the National Wildlife Research Center in Fort Collins, Colorado have reported high survival rates for the rabies vaccine Raboral™ VRG for up to 21 days at or above 37°C after foamation of the virus (Nash et al. in press).

One of the obvious advantages of foamation is that it may eliminate the need to refrigerate the vaccine during storage, shipment and deployment. Currently, biological materials such as live-vector vaccines require low temperatures for stability. However, if degradation of vitrified material does not occur at high temperatures, then refrigeration would no longer be necessary. This would provide options for long-term storage and transport, which would be especially valuable for sensitive biologicals that are used in the field, in underdeveloped areas, or in other situations where refrigeration is not available or transport over long distances is necessary.

1.8 Conclusions

Oral vaccine design requires a multi-disciplinary approach to develop a vaccine with sufficient immunogenicity, stability, and ability to enter the host to induce an appropriate immune response. In addition, oral vaccines may be the only method for controlling over-abundant animal populations through immunocontraception. The studies presented in the following chapters were conducted to determine if a live avirulent *Salmonella choleraesuis* strain 54 (SC54) that expresses a GnRH fusion protein (rmGnRH), delivered to the oral mucosa inductive sites, including the gut and nasal-associated lymphoid tissue, would elicit an immune response against “self” GnRH and

immuno-neutralize CD-1 mice. The need for immunocontraception in addition to protecting the animals against salmonellosis or brucellosis suggests a possible role for a dual oral vaccine. Potential *Brucella suis* vaccine strains VTRS1 and 353-1 (Stoffregan, in press) can be modified to express immunocontraceptive antigens to create potential dual *Brucella*/immunocontraceptive vaccine for feral swine (Kemp and Miller, 2008). Because live vaccines require delivery devices that will protect them from degradation, a novel method for protecting live immunogens was investigated which can stabilize live bacterial vaccines for potential oral vaccination of feral and wildlife species. Using mice, these studies offer a proof-of-concept model for the development of potential oral vaccines for feral and other over-abundant wildlife or pest species.

Chapter II

ENGINEERING THE pNS2TrcD-rmGnRH PLASMID VECTOR AND TRANSFORMATION INTO *SALMONELLA CHOLERAESUIS* STRAIN 54

2.1 Introduction

Heterologous protein expression from *Brucella* bacterial species is of particular interest for brucellosis vaccine research. Recent work has demonstrated that foreign heterologous proteins can be stably expressed from *Brucella abortus* strain 51 (RB51) elicting a protective T-helper cell immune response (Vemulapalli et al., 2000). The plasmid expression vector pNS2TrcD (Figure 2.1) was derived from the host expression vector pNSGroE, a *Brucella* expression vector and has been designed with a Trc promotor, a hybrid of trp-lac (Amann et al., 1983) which shows functionality in *Salmonella*, specifically *choleraesuis spp*. (Seleem et al., 2007b). This particular plasmid was also engineered to be kanamycin resistant and contains three additional sequences; a tandem RNA stem –loop, a UP element, and 6X histidine sequence, which enhance gene expression and allow for easy detection and purification (Seleem et al., 2008). In other words, this particular expression plasmid optimizes the production of a foreign antigen in *Salmonella*, which may improve an immune response to the produced protein.

A 425 base-pair transgene was inserted into the multiple cloning site (MCS) of the expression plasmid pNS2TrcD (Seleem et al., 2008). The resulting expressed protein was predicted to be 16-18kDa and consisting of five gonadatropin releasing hormone (GnRH) decapeptides sequences (Figure 2.2) interspersed with four T cell

epitopes from various disease causing pathogens including *Plasmodium falciparum*, *Mycobacterium tuberculosis*, tetanus toxin, respiratory synctial virus (RSV), and measles (Gupta et al., 2004). Immunologically, T cell epitopes should assist in promoting an immune response to hormones which are otherwise "self" molecules and to which the body is normally immunologically tolerant (Talwar et al., 2004).

In 2005, a sample of purified rmGnRH protein was sent to scientists at the National Wildlife Research Center (NWRC) to begin working on methods of immunocontraception in wildlife or feral animals. The protein was hydrated and mixed with AdjuVac™ and 500µg of rmGnRH was injected intramuscularly into mature gilts. An immunocontraceptive effect was observed in 3 of 5 gilts after a single injection (Miller et al., 2006). The DNA encoding rmGnRH (Figure 2.3) was then sent to the NWRC to assist in production of the rmGnRH protein. During this time, communication with Dr. Stephen Boyle at Virginia Polytechnic Institute highlighted the desire of expressing the rmGnRH gene in Brucella species. These collaborative efforts produced a series of sub-cloned *Brucellae/Salmonella*e expression plasmids containing the rmGnRH genetic sequence, one being the pNS2TrcD-rmGnRH plasmid.

The selected carrier bacteria for the plasmid was *Salmonella choleraesuis* strain 54 (SC54), which was provided by Dr. Michael Roof (Boehringer Ingelheim, Ames, Iowa). SC-54 (Enterisol-54®) is an avirulent live strain of bacteria registered by the USDA for intranasal and oral vaccination to prevent salmonellosis in swine, which is caused by *S.choleraesuis* (Fox et al., 1997). SC-54 has been tested in pigs (Letelleier et al., 2001; Maes et al., 2001) and cattle (Hermesch et al., 2008) and shows protective efficacy against a virulent challenge. In addition, calves given oral SC54 experienced a

reduction of clinical signs of salmonellosis after a *Salmonella dublin* challenge (Fox et al., 1997).

The objective of this experiment were to sub-clone the rmGnRH transgene sequence into the compatible gram-negative bacteria expression plasmid pNS2TrcD, transform the resulting plasmid into *Salmonella choleraesuis* strain 54 and confirm expression of the fusion protein.

2.2 Materials and Methods

2.2.1 Plasmids and DNA manipulations

DNA ligation and restriction endonuclease digestions were performed according to standard techniques (Sambrook, 1989) and were performed in Dr. Stephen Boyle's laboratory (Virginia Polytechnic Institute, Blacksburg, VA). Polymerase chain reactions (PCR) were performed using Platinum PCR Super Mix High Fidelity (Invitrogen, Carlsbad, CA, USA) and a Gradient Mastercycler® (Eppendorf; Hauppauge, NY). Oligonucleotides were purchased from Sigma-Genosys (Sigma-Aldrich; St. Louis, MO, USA). Restriction and modification enzymes were purchased from Promega (Madison, WI). QIAprep Spin Miniprep Kit from QIAGEN (Valencia, CA, USA) was used for all plasmid extractions and QIAGEN PCR cleanup kit was used for removal of restriction enzymes and DNA gel extraction. For all selection and growth methods, kanamycin was used at a final concentration of 50µg/ml.

2.2.3 Construction of pNS2TrcD-rmGnRH

The rmGnRH DNA sequence was amplified from XXXX using primers GnRHF-2 and GNRHR-R (Table 2.2). *BamHI* and *Spe*I restriction sites were incorporated in the forward and reverse primers for directional cloning. Restriction digestion, gel

purification, and ligation with plasmid pNS2TrcD was performed with temperature cycle ligations method (TCL) as described by (Lund et al., 1996) using a Mastercycler® Gradient programmed to cycle indefinitely between 30s at 10ºC and 30s at 30ºC for 1 hour to form pNS2TrcD-rmGNRH. Confirmation of the insert (GNRH) was performed by PCR using the primers GnRHF-2 and GNRHR-R (Table 2.2).

2.2.4 Agarose gel electrophoresis of pNS2TrcD-rmGnRH

The pNS2TrcD-rmGnRH plasmid was screened using PCR and its size verified using agarose gel electrophoresis. All procedures were performed according to standard techniques (Sambrook J, 1989). Briefly, 10µl of each DNA sample was loaded onto a 0.7% agarose gel and was run at 70V for 1 hour. Ethidium bromide was used to visualize the DNA bands representing the molecular weight ladder and 3 replicate constructs.

2.2.5 Transformation of pNS2TrcD-rmGnRH into Salmonella choleraesuis strain 54

Lyophilized *Salmonella choleraesuis* strain 54 (SC-54) was rehydrated according to the manufacturer's recommendations with 100 ml of sterile deionized water provided and allowed to incubate at room temperature for 10 minutes. Preparation of competent SC54 followed previously published protocols (Sambrook J, 1989). Briefly, SC54 was grown overnight in 100 ml of 2xYT broth at 37°C. A fresh 100 ml aliquot of 2xYT was inoculated with 1 ml of theovernight culture and incubated at 37°C with shaking at 250rpm until the culture reached an optical density of 0.4 at 600nm. The bacterial cells were placed on ice for 20 minutes and centrifuged at 1300 x g for 10 minutes in 50ml conical vials. The cells were then resuspended in 30mls of 0.1M $CaCl_2$, incubated on ice for 30 minutesk, centrifuged again at 1300 x g for 10 minutes and re-suspended in 30mls of 0.1M $CaCl_2$ solution. A 1ml aliquot was centrifuged and the 0.1M $CaCl_2$ was

discarded. Twenty microliters of pNS2TrcD-rmGnRH was added to the cell suspension and incubated on ice for 30 minutes. After incubation, 400µl of 30°C SOC media (Invitrogen; Carlsbad, CA USA) was added and the cell suspension incubated at 37°C for 1 hour. The cells were then inoculated onto kanamycin selection plates and incubated overnight at 37°C. Transformed colonies were isolated, added to 100 ml of fresh 2xYT with 50 µg/ml kanamycin, and incubated for 5 hours to make stocks.

2.2.6 SDS-PAGE

A 6-hour protein induction experiment using the transformed SC54 was performed to confirm that rmGnRH was being expressed by the bacteria. A 1 ml aliquot from 100ml 2xYT broth was obtained as the pre-induction sample. Expression from the trc promoter was then initiated by adding 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture, and 1 ml aliquots were removed at 2 hour intervals. Each of the five 1ml samples were centrifuged in 1.5ml microcentrifuge tubes at 14,500 x g for 5 minutes and washed by re-suspending the cells with sterile saline. This step was repeated two additional times. After the final centrifugation, the cell aliquots were re-suspended in 50µl of Tris-HCl and 50µl of 2X Laimelli sample buffer and incubated at 95°C for 5 minutes. The samples were again centrifuged at 14,500 x g for 5 minutes to remove any remaining cellular debris and the supernatants analyzed by SDS-PAGE. The SDS-PAGE gel concentration was a 4-12% gradient, and 20µl of each prepared sample was loaded on the gel and electrophorsed at 80V for roughly 60 minutes, until the protein standard ladder reached the bottom of the gel.

2.2.7 Immunoblot Analysis

The proteins in the gel were transferred to a nitrocellulose membrane using a Transblot SD electrotransblotter (BioRad; Hercules, CA) at 100V for approximately 60

minutes. The nitrocellulose membrane was then stained with Imperial Protein stain solution to confirm protein transfer. Next, the membrane was blocked using 1% milk in 1X PBS for 30-40 minutes. The membrane was probed overnight at 5°C with a primary antibody rabbit anti-rmGnRH (1:1,000).. The primary antibody was removed and the membrane was washed 3 times with 1X PBS/Tween. The secondary labled antibody AG-HRP (1:5,000, Invitrogen), was added and incubated at room temperature for 60 minutes. The membrane was then washed with 1X PBS/Tween and a peroxidase solution was applied to visualize the proteins. As a control and to determine that the 6X histidine fusion tag was present on the protein, a duplicate immuno-blot was performed using a primary mouse anti-histidine antibody (1:1000, Invitrogen) and secondary rabbit anti-mouse HRP antibody (1:5000; Sigma). All washing and incubation steps were kept consistent.

2.3 Results

2.3.1 Construction of the plasmid vectors

The molecular weight of rmGnRH was observed at ~500bp. Confirmation of the transgene rmGnRH was obtained by PCR using primers GnRHF-2 and GNRHR-R and agarose gel electrophoresis (Figure 2.4).

2.3.2 Agarose gel electrophoresis of pNS2TrcD-rmGnRH

The size of the expression plasmid pNS2TrcD-rmGnRh was observed to be ~3500 bp (Figure 2.5). Three independent clones of pNS2TrcD-rmGnRH were analyzed by agarose gel electrophoresis and compared against a broad range molecular weight standard (BioRad; Hercules, CA).

2.3.3 SDS-PAGE rmGnRH protein expression confirmation

A clear increase in expression of a 19kDa protein was observed over the 6 hour time course. However, no increase in general band density was observed after 4-5 hours of induction time (Figure 2.6).

2.3.4 Immuno-blot Analysis

Immunoreactivity of the expressed proteins was visualized using immuno-blot analysis. Two different indicator antibodies were used to determine the presence of the desired epitopes. Positive reactivity against rmGnRH antibody in the 2, 4 and 6 hour time points were present (Figure 2.7). Mouse anti-histidine antibody was clearly present in the 2, 4, and 6 hour time points (Figure 2.8).

2.4 Discussion

The objective of this experiment was to clone the 500 bp transgene rmGnRH into the *Salmonella* compatible pNS2TrcD expression plasmid and to show protein expression from transformed SC54 when the bacteria were induced. The steps taken yielded a stable pNS2TrcD-rmGnRH expression plasmid carrying a kanamycin resistance gene, an A-rich UP element, and an RNA stem loop sequence that improve overall protein expression (Seleem et al., 2008). These additional modifications for protein expression may have been the reason for the general plateau of protein concentration seen after 4 hours of induction. Unfortunately, the kanamycin resistance selection gene makes the use of this particular expression plasmid and bacterial modification in SC54 limited to only laboratory models and proof-of-concept studies in a controlled setting. However, recently developed attenuated *Brucella* and *Salmonella* compatible auxotrophic plasmids may offer an opportunity for use in field settings (Poester et al., 2006; Seleem et al., 2006).

Using auxotrophic plasmids and/or bacteria will also help to mitigate an immune response in any secondary target animals. If a secondary target were to ingest the modified Salmonella, the animal would also need to consume the supplemental dietary substrate for any bacterial protein expression to take place. Ideally, oral live vaccines would be delivered to animals in a mechanized species specific device, where the substrate necessary for protein expression would only be available to the specified target. Species specific devices are being tested for development of pig specific vaccines (Campbell et al., 2011).

Immunoreactivity of the rmGnRH protein from SC54 observed in lanes 2, 3, and 4 on the SDS-Page gel were reactive against a previously prepared rabbit anti-rmGnRH antibody using immuno-blot. The analysis also shows that a relatively high level of cross-reactivity against other antigens from SC54 were present. A possible reason for this cross-reactivity may be due to the interspersed T cell epitopes located within the protein. As a control, the immunoreactive presence of the fused histidine tag located on the N-terminus of the expressed protein was observed, as well as overall immunoreactive function against rmGnRH antibody. The upstream his-tag sequence addition to the pNS2TrcD plasmid provides a useful tool for purifying the expressed protein and for potentially scaling-up *in vitro* protein production methods. The histidine tag has high affinity to metal ions like nickel (Ni+2) that can be adhered to resin in chromatography column. Cell lysate containing his-tagged proteins are bound to the Ni+ on the column and later eluted off and then concentrated. Verifying the presence of a his-tag sequence supports the claim that the protein was correctly expressed from the bacteria.

Salmonella choleraesuis strain 54 has been patented for use to induce an immune response in animals and the method for making the strain avirulent has also been patented

(Kramer, 1995, 1996). SC54 has been highly successful in electing a protective immune response against challenge in numerous studies. SC54 has not been found to be shed in the feces of bovine treated with it and there is no evidence of reversion after laboratory and field evaluations (Kramer, 1995; Letellier et al., 1999). For these reasons, SC54 is an obvious candidate to use as an oral bacterial vector to express foreign heterologous antigens.

2.5 Conclusion

This study was used as a partial proof-of-concept for the potential development of oral immunocontraceptive vaccines. A practical wildlife vaccine that uses live bacteria to express a foreign antigen will lack any antibiotic resistance. Instead, it will have to rely heavily on auxotrophic attenuation, making the bacterium dependent on a dietary substrate not normally found in a natural setting. This will be the safest means for regulating the protein expression of the bacteria composing the oral vaccine. These experiments have shown the successful expression of a multimeric GnRH fusion protein from the *Salmonella choleraesuis* strain 54.

2.6 Tables

Table 2.1. Description of the *Salmonella* compatible expression plasmid pNS2TrcD.

Plasmid	**Description** [a]	**Reference**
pNS2TrcD	Kan^r, Expression vector using hybrid *TrcD* promoter	(Seleem et al., 2007a)

[a] Kan^r, Kanamycin resistance.

Table 2.2. Primers used for PCR amplification of the rmGnRH genetic sequence.

Primers	Primer sequence
GNRH-F2	5`-CGACGATGACGATAAGGATCCGAATTCATG-3`
GNRH-R	5`- GGGACTAGTCTTCGAATTCCATGGTACCAGCTG-3`

2.7 Figures

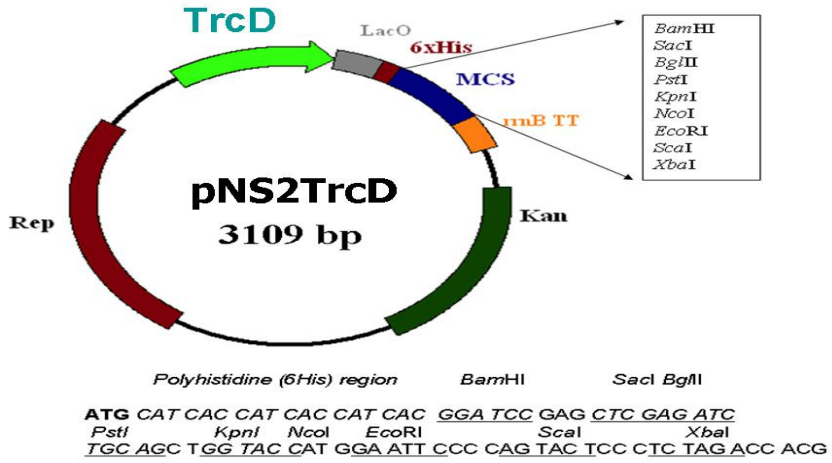

Figure 2.1. pNS2TrcD Plasmid Map
The engineered pNS2TrcD expression vector for regulated expression with the His-tag fusion in the N-terminus (Seleem et al., 2007a); MCS, multiple cloning site; kan, kanamycin gene conferring kanamycin resistance; *rep*, gene required for plasmid replication; 6Xhis, His tag fusion; LacO, lac operator for regulated expression.

GnRH *Plasmodium falciparum*
pE-H-W-S-Y-G-L-R-P-G//-D-I-E-K-K-I-A-K-M-E-K-A-S-S-V-F-N-V-V-N-S//-

GnRH Tetanus toxoid
E-H-W-S-Y-G-L-R-P-G//-Q-Y-I-K-A-N-S-K-F-I-G-I-E-L//-

GnRH Respiratory syncytial virus
E-H-W-S-Y-G-L-R-P-G//-A-E-Y-N-V-F-H-N-K-T-F-E-L//-

GnRH Measles virus
E-H-W-S-Y-G-L-R-P-G//-L-S-E-I-K-G-V-I-V-H-R-L-E-G-V//-

GnRH
E-H-W-S-Y-G-L-R-P-G

Figure 2.2. Amino Acid Sequence of rmGnRH showing five repeating gonadotropin releasing hormone (GnRH) sequences interspersed with T-cell epitopes from *P.falciparum*, Tetanus toxoid, RSV, and measles.

ATGACTG GTGGACAGCA AATGGGTCGG GATCTGTACG
ACGATGACGA TAAGATCCCC GAATTCATGG AACATTGGAG CTATGGCCTG
CGTCCGGGCG GTAGCGGTGA TATCGAAAAA AAAATCGCGA AAATGGAAAA
AGCGAGCAGC GTGTTTAACG TGGTGAACGG TAAGCTTAGC GGAGAACATT
GGAGCTATGG CCTGCGTCCG GGCAGCGGTG CGGAATATAA CGTGTTTCAT
AACAAAACCT TTGAACTGCC GCGTGCGGGT GGTGAACATT GGAGCTATGG
CCTGCGCCCG GGCGGTGGTC AGTATATCAA AGCGAACAGC AAATTTATCG
GCATTACCGA ACTGGGTAGC GGTGAACATT GGAGCTATGG CCTGCGTCCG
GGCGGTAGCG GTCTGAGCGA AATCAAAGGC GTGATCGTGC ATCGTCTAGA
AGGCGTGGGT AGCGGTGAAC ATTGGAGCTA TGGCCTGCGT CCGGGCTAAT
AG

Figure 2.3. The genetic sequence of rmGnRH (425bp). Green shaded areas correspond to start and stop codons, while the yellow shaded areas represent the five interspersed GnRH sequences.

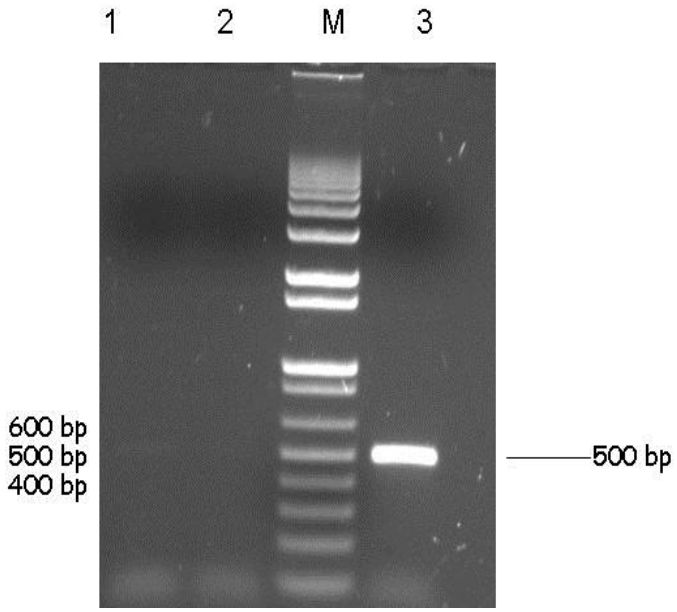

Figure 2.4. Ethidium bromide stained PCR confirmation of the rmGnRH transgene insert, 0.7% Agarose gel electrophoresis, 1-3: different constructs that were screened by PCR using primers GnRHF-2 and GNRHR-R. The construct located in lane 3 verifies the correct size (bp) of rmGnRH. Molecular weight (MW) ladder is located in lane M.

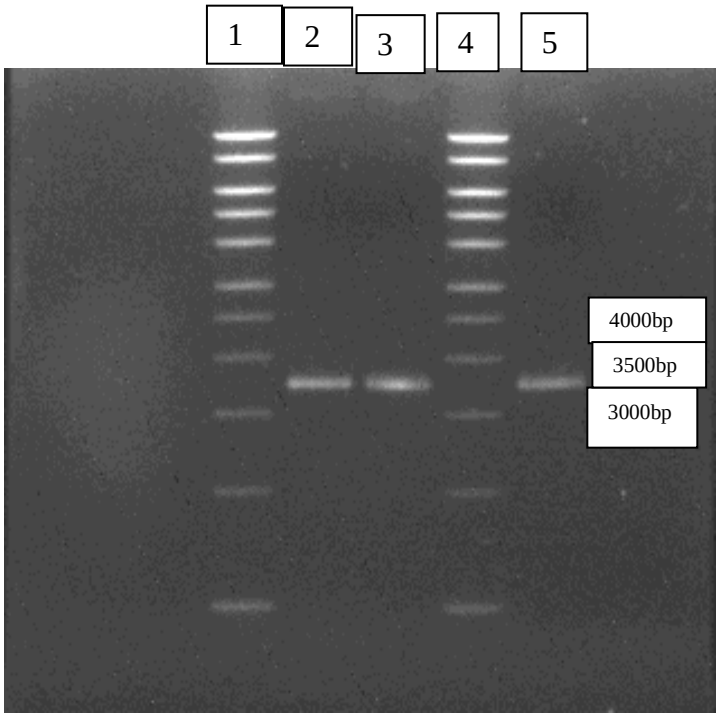

Figure 2.5. Agarose Gel Electrophoresis confirming pNS2TrcD-rmGnRH. 0.7% Agarose gel electrophoresis , lanes 2,3,and 5 are replicate constructs that were screened by PCR using primers GnRHF-2 and GNRHR-R showing a molecular weight of ~3500bp. Molecular weight was determined in lanes 1 and 4 using a broad range (MW) ladder. Ethidium bromide was used to visualize the bands.

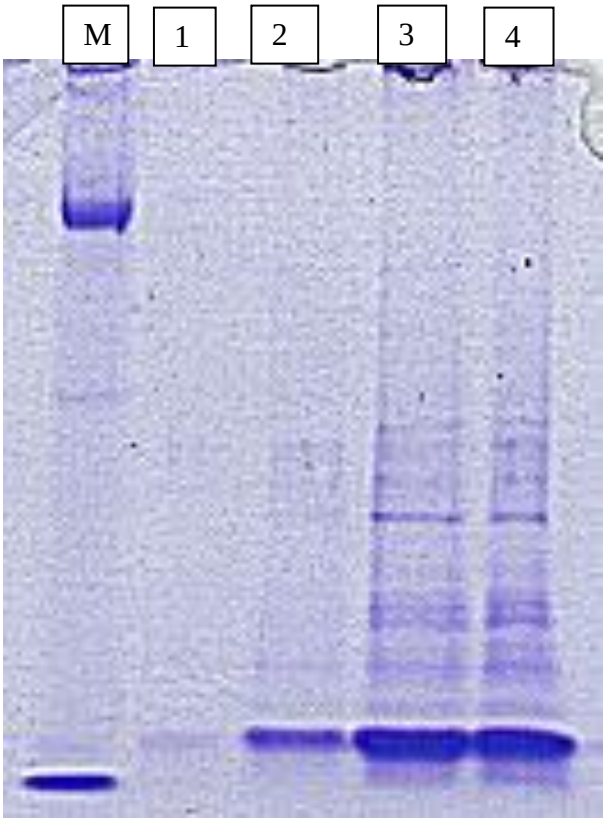

Figure 2.6. SDS-PAGE Gel over-expressed rm-GnRH with 6hr protein inductions from modified SC54. Aliquots were taken at 0, 2, 4, and 6 hour intervals (lanes 1-4). Molecular weight of rmGnRH was verified between 16-18kDa. Molecular weight marker (BioRad).

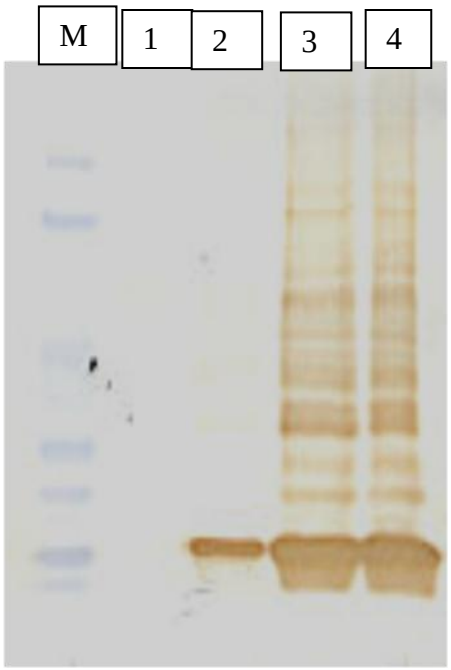

Figure 2.7. Immuno-blot rmGnRH protein induction from modified SC54. Primary antibody used was rabbit anti-rmGnRH (1:1000) and secondary antibody protein AG-HRP (1:5000). Molecular weight (MW) marker is from BioRad, located in lane M. two hour time point aliquots are located in lanes 1-4.

Figure 2.8. Immuno-blot analysis of rmGnRH protein induction from modified SC54.Primary antibody used was mouse anti-histidine (1:1000) and secondary antibody protein AG-HRP (1:5000). Molecular weight (MW) marker is from BioRad located in lane M. Two hour time point aliquots are located in lanes 1-4.

Chapter III

ORAL DELIVERY OF RECOMBINANT SC-54

3.1 Introduction

A growing need to develop non-lethal means for controlling over-abundant wildlife populations has encouraged research into immunocontraceptive technology. Numerous studies have shown that contraceptive vaccines can be effective in inhibiting reproductive cycling in horses (Kirkpatrick et al., 1992), dogs (Bender et al., 2009; Massei et al., 2010), cats (Levy et al., 2004), prairie dogs (Yoder et al., 2008), and white-tailed deer (Gionfriddo et al., 2009; Gionfriddo et al., 2011). The species used in those previous studies represent animals that pose a risk to human health through possible infectious disease transmission due to over-population or are considered pests in various regions around the world. Lethal control through culling animals has been used when a reduction in animal numbers is required for wildlife management. However, the use of non-lethal immunocontraceptive technologies may offer an alternative or an additional tool for managing wildlife or free-ranging animals.

Immunocontraceptive vaccines are directed against self, reproductive antigens, either hormones or proteins, to which the recipient is normally immunologically tolerant (Amman et al., 1983). Traditionally, immunocontraceptive vaccines have been designed to cause an immune response against “self” proteins derived from sperm or zona pellucida (ZP) or by making antibodies against GnRH which neutralizes circulating GnRH, interfering with normal downstream regulation of the gonadatropes

(Thompson Jr., 2000). In addition, in males, GnRH neutralization by vaccines results in decreased testicular weights and testosterone levels (Levy et al., 2004; Ulker et al., 2009).

There has been great success with using parenteral immunocontraceptive vaccines in various wildlife and domestic animal species. However, the high financial costs associated with capture and handling of free ranging animals for vaccination makes it cost prohibitive and impractical for broad-scale field use. Although parenteral immunocontraceptive vaccines are effective in certain controlled settings, the only practical approach to vaccinate free ranging animals is by oral immunization.

One method for developing oral immunocontraceptives is through modified avirulent live organisms, such as bacteria. Many non-bacterial antigens can be expressed by gram-negative bacteria, including *Escherichia coli* (*E.coli*) and *Salmonella*. In addition, these organisms have the innate ability to invade the mucosal epithelial layers of the gastrointestinal and nasal-associated lymphoid tissue making them an obvious choice for development of oral vaccine development. Several modified avirulent bacteria have been developed that express a mammalian "self" reproductive protein or antigen (Gupta et al., 1997; Skinner et al., 1994) and these may provide an approach for developing effective oral immunocontraceptive vaccines (Miller et al., 2000). Additionally, numerous GnRH fusion proteins have been developed that contain immunologically well-known T cell sequences that induce enhanced antibody production. Interestingly, some of this research has been mainly driven by the need to develop better treatments against prostate cancer in humans by decreasing testosterone levels (Talwar et al., 2004).

When a recombinant multimeric gonadotropin releasing hormone (rmGnRH) fusion protein was injected intramuscularly (IM) to reproductively mature pigs one time with AdjuVac™ as an adjuvant, GnRH neutralization occurred with downstream

immunocontraception for 6 months. Titers were measured to be near 128,000 one month after the initial injection, well beyond the accepted immunocontraceptive threshold of 64,000 (Miller et al., 2006). That recombinant protein consists of five GnRH sequence residues interspersed with four T-cell epitopes, and when folded post-translationally, the five GnRH peptide sequences are localized on the surface of the protein, and the T-cell sequences localize in the interior of the protein (Gupta et al., 2004). The unique design and folding pattern of this "fusion" protein elicits an impressive host immune response and iummunocontraceptive effect in pigs. By neutralizing GnRH, the downstream production of luteinizing hormone (LH) is interrupted, causing a decrease in testosterone production and ultimately atrophy in testosterone tissue (Talwar and Sad, 1989).

Zoonotic and avirulent bacteria, such as RB51, are routinely used as vaccines to protect cattle against brucellosis (Vemulapalli et al., 2000). *Salmonella choleraesuis* strain 54 (SC-54) is an avirulent live culture, registered by the USDA, for intranasal and oral vaccination, to aid in the prevention of salmonellosis in swine caused by other virulent *Salmonella* species (Fox et al., 1997). The DNA sequence encoding for rmGnRH was cloned into pNS2TrcD, an expression plasmid engineered for enhanced protein expression in *Salmonella* and *Brucella* bacterium species (Seleem et al., 2007b). *Salmonella choleraesuis* SC-54 was then made recombinant by inserting or transforming the pNS2TrcD expression plasmid containing the rmGnRH DNA sequence (pNS2TrcD-rmGnRH) into the bacterium resulting in a modified or recombinant SC54 (rSC54). As a result the bacterium retains all of the normal vaccine properties, but produces the additional rmGnRH protein. The target tissues of bacterium include the mucosa-associated lymphoid tissue found in the intestine and nasal areas of mice, and it is possible that if sufficient antibodies are generated against the rmGnRH protein expressed

from the rSC-54, a host immune response will occur, thereby eliciting an immunoneutralization effect.

Our experiments were aimed at determining if an antibody response could be generated against "self" GnRH in mice when recombinant *Salmonella choleraesuis* strain 54 expressing a GnRH fusion protein are delivered mucosally. In addition, mice were given rSC54 parenterally to compare routes of administration and determine if a similar immune response can be elicited.

3.2 Materials and Methods

3.2.1 Bacterial vector

Salmonella cholereasuis (SC-54) was donated by Dr. Michael Fox at Boehringer Ingelhiem, Vetmedica in Ames, Iowa.

3.2.2 Expression plasmid vector (pNS2TrcD)

The expression plasmid pNS2TrcD was donated by Drs. Stephen Boyle and Mohammed Seleem at Virginia PolyTechnic University, Center for Molecular Medicine and Infectious Diseases (CMMID) in Blacksburg, Virginia. This expression vector for S*almonella* spp., consists of the pNS backbone and is derived from the broad host range cloning vector (plasmid) pNSGroE (Seleem et al., 2008), and a regulated TrcD promoter from *Brucella abortus*. The plasmid confers kanamycin resistance for regulated growth, a RNA stem loop and UP element for enhanced gene expression. The plasmid also attaches a 6X histidine sequence to the N-terminal on the expressed protein for simplifying protein purification and collection using nickel affinity chromatography.

3.2.3 Transgene and Expressed multimeric Protein (rmGnRH)

The multimeric gonadotropin releasing hormone (rmGnRH) is a 16-18 kDa fusion protein consisting of 5 GnRH genetic sequences interspersed with 4 immunologically

"well known" T cell sequences. The T cell sequences are derived from various zoonotic infectious diseases and are meant to enhance an immune response once exposed to an immune system. The protein is expressed from the bacteria with a 6X histidine tag on the N-terminal for purification and collection. It is theorized that the tertiary structure of the protein places the 5 GnRH peptide sequences on surface of protein.

3.2.4 Preparation and Growth Conditions of rSC54

Previously prepared glycerol stock cultures of rSC54, generated by recombinant DNA techniques as described in chapter two of this dissertation were thawed at room temperature. Fresh 2xYT broth (100mls) was inoculated with 20µl of thawed rSC54 and placed into an incubator/shaker grown overnight at 37°C and 250rpm. Then, 1ml of over-night rSC54 culture was transferred to 100mls of fresh 2xYT both and grown to an OD of 0.4 at 600nm. The bacteria were then induced with 1mM IPTG and allowed to express the rmGnRH protein for 5 hours. The bacteria were then centrifuged at 10,000 X g for 4 minutes, and re-suspended into phosphate buffered saline (PBS) to approximately $7X10^8$ cells/ml of rSC54. These steps where repeated 30 days later when a dose of $2X10^9$ cells/ml rSC54 was prepared for a boost. In a separate beaker, non-modified SC54 was grown with the same overnight conditions, centrifuged at 10,000 X g for 4 minutes, and was re-suspended into PBS to approximately $3.4X10^8$ cells/ml. The density of the bacteria was adjusted by optical density measurement at 600nm, and confirmed by serial dilution and plate counting.

3.2.5 Animals and Treatments

Sixty-one CD-1 mice at 4 weeks of age were randomly assigned to seven test groups. Route of administration was tested and treatment groups consisted parenteral delivery and mucosal delivery of recombinant SC54 (rSC54). Mice were placed in to the

following treatment groups; control (n=7), rSC54 oral gavage (n=9), rSC54 nasal lavage (n=9), subcutaneous injection (SC) of heat-killed rSC54 (n=9), subcutaneous(SC) injection of live rSC54 (n=9), intramuscular injection (IM) of 25µg/50µl purified rmGnRH protein emulsion (n=9), and oral gavage of non-modified SC54 as a control (n=9). Oral treatment animals received 20µl of bicarbonate/ carbonate (100mg/ml) oral gavage bolus prior to treatment for de-acidification of the stomach contents. Animals in mucosal delivery treatment groups (oral and nasal) were immunized with 0.2mls of $7X10^8$ cells/ml r SC54 cell suspension, with a boost 30days later of $2X10^9$ cells/ml rSC54. Animals that received a parenteral delivery were subcutaneously injected with the same concentration of rSC54 ($7X10^8$) in the same volume of PBS (0.2ml). Control animals that received non-modified SC54 were delivered by oral gavage $3.4X10^8$ cells/ml. No boost was given to this control group.

3.2.6 Data Collection

3.2.6.1 Serum Collections

All animals were bled via tail vein puncture with a 25-27 gauge needle, or by making a small cut at the same site and collection of blood using capillary tubes at 1month intervals throughout the experiment, beginning prior to immunization??. Blood samples were centrifuged at 7000 X g for 15 minutes, and serum was harvested and stored at -20°C until subsequent analysis.

3.2.6.2 Enzyme-linked Immunosorbant Assay (ELISA)

Enzyme linked immunosorbant assay was used to determine the overall immune response to GnRH. Sera were serially diluted from 1/1000 to 1/12800 in 96 well immunoassay plates coated with 200ng of bovine serum albumin –GnRH (BSA-GnRH) conjugate (NWRC) and incubated overnight at 5°C. The plates were then blocked

overnight with 5% Tween/20%SeaBlock (Pierce)/ in PBS blocking solution. A secondary mouse anti-rabbit horse-radish peroxidase antibody diluted 1/4000 was used to analyze for the presence of mouse anti-rmGnRH antibodies. The antibody titers were measured as the A_{450} of the serum dilutions. Using an Excel (Microsoft) macro, the titers were calculated for each treatment sample and corresponding date using the pre-bleed sample as the comparison value. Absorbance values were calculated by subtracting the detection limit value from each sample value.

3.2.6.3 Tissue Weights, Histology, and Spermatozoal Motility

Upon completion of the study and euthanasia of animals, both testes were collected from each mouse to observe if weight and morphological change occurred. Testes was rinsed with sterile water and dried by dabbing on Kim wipes until the outer surface was dry. In addition, both epididymides were excised and were evaluated for the presence of viable spermatozoa. The testes were nicked with a razor blade at one end of the tunica albuginea to allow optimal fixation in Bouin's solution. Both testes and one epidiymus from each animal were fixed in Bouin's solution for 2 days, then washed three times and stored using 70% ethanol. The tissues were processed for histological examination and evaluated histologically. Six micrometer thick sections of testis and epididymus from paraffin embedded samples were stained with hematoxylin and eosin stains, mounted and examined for sperm and morphological changes. Photomicrographs were taken under a light microscope (Lieca Microsystems, Buffalo Grove, Illinois, USA). Sperm were collected as described previously (Dewit et al., 2000). Briefly, one epididymis was massaged with a tweezers to expel sperm from within the tissue and equilibrated in 0.5ml TALP solution for 20 minutes. To determine the percentage of motile spermatozoa in a sample, a 6µl sample of diluted spermatozoa from each animal

was placed onto a Cell-Vu glass slide (Fertility Technologies, Inc. Natick, MA, U.S.A) and the percentage of motile spermatozoa was determined using a computer-assisted sperm analysis instrument (CASA) model (IVOS, Hamilton-Thorne, Beverly, MA, USA), with the warming stage set to 37°C. The following parameters were used for analysis: ≥200 spermatozoa per sample, 30 frames collected at 60Hz; minimum contrast 75; minimum cell size 8; non-motile head size 25; non-motile head intensity 75; non-motile minimum contrast 15; motile cells must travel >3μm/s. In addition, a visual total and progressive percentage value was measured by placing 6μl of diluted spermatozoa sample on a glass slide and visually observed and measured by light microscope. Recorded values for this experiment include CASA total and progressive, visual total and progressive, and concentration of sperm in the millions (M).

3.3 Statistical Analysis

Treatment differences for the tissue weights and sperm cell numbers were analyzed usinganalysis of variance (ANOVA; SAS Institute Inc., 2011).

3.4 Results

Antibody titers for rmGnRH were negative in pre-immunization samples and in all samples taken from untreated animals. Antibody titers were greatest four weeks after immunization in the oral gavage group and animals that received purified rmGnRH as an injection ($P<0.05$; Figure 3.1), although titers for both the nasal lavage group and animals that received a SC injection of live rSC54 showed a delayed increase in antibody response after 8 to 16 weeks ($P<0.05$). Likewise, a reduced testis size was observed in mice that were orally immunized with rSC54 and mice that were injected IM with purified rmGnRH ($P<0.05$; Table 3.1; Figure 3.2). Epididymal weights showed no significant difference between treatment and control animals, as well as no measureable

difference in cell motility characteristics. However, a reduced sperm cell concentration was observed between orally treated mice and the IM positive control group when compared against non-treated control mice ($P<0.05$; Table 3.1; Figure 3.3).

3.4.1 Pathology

No significant morphological change was observed in treated or control mice. Testis weight and sperm concentration was decreased in groups 1, the oral gavage treatment group, and 5, the positive control treatment group, during treatment, but those morphological differences could not be observed visually (Figure 3.4).

3.5 Discussion

A recombinant avirulent *Salmonella choleraesuis* strain 54 (SC54), constructed by inserting a compatible expression plasmid pNS2TrcD containing the transgene rmGnRH was used as a vector for the expression of heterologous GnRH fusion proteins and to orally vaccinate mice. Immunogenicity of the expressed rmGnRH from SC54 was determined previously by Western blot and sufficient concentrations of protein were expressed as visualized by SDS-PAGE analysis (Chapter 2). Generally, immune response against heterologous expressed proteins from bacteria can be influenced by where the protein or antigen is being expressed by the bacterium, either intracellularly or extracellularly. Because this protein is not combined to any surface protein sequences, it is expressed intracellularly and accumulates in the cyloplasm of the bacteria. Although secreted antigen display has been found to be more effective in eliciting a protective immune response (Kaufmann and Hess, 1999), a somatic antigen expression also can induce a delayed, but protective immune response. Most protective antigens, expressed through bacterial vectors, from viruses and parasites have been expressed in the form of fusion proteins which accumulate in the cytoplasm (Dougan et al., 1987). Proteins that

are secreted by bacteria within the phagosome or cytoplasm are readily introduced into the appropriate MHC processing machinery. Somatic antigens are encapsulated within bacteria and temporarily shielded from antigen processing. Not until bacterial pathogens are killed and degraded, do somatic cytoplasmic antigens become accessible for MHC processing (Kaufmann and Hess, 1999). Following this logic, bacteria that were killed during delivery to the gastrointestinal lymphoid tissue may have expedited the presentation of antigens to T cells by bypassing any internal phagosomal residence time. Those cells that were delivered to nasal-associated lymphoid tissue may have exhibited the delayed antigen presentation due to modified SC54 remaining within the phagosome. It must noted that the maximum titer measured in the oral gavage treatment group was 12,800, which, although may be immuno-neutralizing the animals, did not reach a threshold titer of 64,000 or higher, which is known to be an accepted antibody titer value required to immunocontracept most mammals using specific GnRH conjugate vaccines. Whereas, the positive control group that received an IM injection of purified rmGnRH resulted in ELISA titers greater than 12800. A breeding study is needed to determine the actual immunocontraceptive effect of orally delivered rSC54, and intramuscularly injected purified rmGnRH. Mice that received subcutaneous injections of either live or heat-killed modified SC54 did not exhibit a significant immune response, indicating that this bacterial strain must be delivered to a mucosal surface for adequate antigen presentation and immune response to occur.

3.6 Conclusion

This experiment reveals that an adequate immune response against “self-GnRH” antigens relies on the location of antigen expression and mucosal lymphoid region that the antigen is delivered. Also, this experiment shows that an immune response against an

immunocontraceptive antigen can be elicited from *Salmonella choleraesuis* strain 54 when delivered orally or nasally. Although an immunocontraceptive antibody threshold value may have not been reached in any of the animals, a discernable immune response was observed in animals indicating that the expressed rmGnRH protein was successfully exposed to the mouse immune system. Additional studies utilizing oral bacteria as delivery vectors for immunization is needed to help better understand the mechanisms involved in oral vaccine delivery.

3.7 Tables

Table 3.1. Tissue weights and spermatozoa motility measurements of mice after treatment with rSC54 delivered orally, nasally, and subcutaneous injection. Controls included an intramuscular injection (IM) of purified rmGnRH emulsion, non-modified SC54, and mice that received no treatment. A reduced sperm count and testis weight was observed in positive control mice and animals that were orally gavaged compared to No Treatment control group ($P<0.05$). No significant differences between other treatment groups were observed (n=9).

Treatment Con.	Testis (g)	Epididymus (g)	CASA Tot. (%)	CASA Prog. (%)	V (%)	isual Tot. (%)	Visual Prog. (10^6)
Oral Gavage	0.1[a]	0.054	50	14	48	22	715[a]
Nasal Lavage	0.125[b]	0.048	45	23	59	25	1837[b]
Live (Sc)	0.122[b]	0.05	64	22	55	20	2022[b]
Dead (Sc)	0.128[b]	0.059	53	19	48	23	3898[b]
Purified rmGnRH (IM)	0.086[a]	0.065	63	25	57	31	398[a]
SC54	0.145[b]	0.068	33	16	37	23	3625[b]
No Treatment	0.146[b]	0.056	67	27	71	34	5198[b]
SEM	0.006	0.007	9	5	9	6	698

Within a column, values with different superscripts (a and b) are different ($P<0.05$).

3.8 Figures

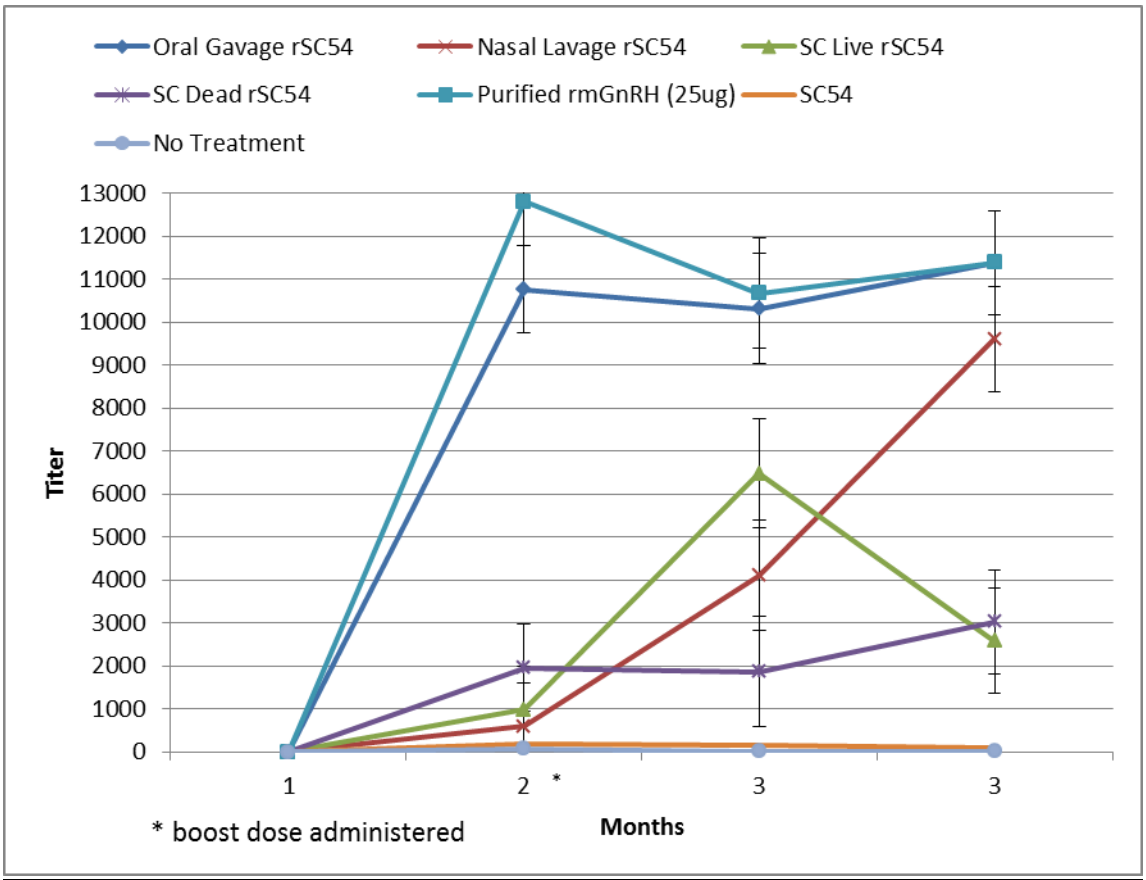

Figure 3.1. Titer values (+/- SE) representing anti-rmGnRH antibody response for each for treatment group 1 (oral gavage), 2 (nasal lavage), 3 (live rSC54 subcutaneous injection), 4 (dead rSC54 subcutaneous injection, positive control group 5 (intramuscular injection of purified rmGnRH protein, 6 (oral gavage of non-modified SC54), and 7 (no treatment control). Oral gavage group 1 and positive control group 5 were similar with increased titer when compared to control group 6 or non-treated animals in group 7 ($P<0.05$). Animals were boosted two days after the 1/21/2011 blood collection. (n=9)

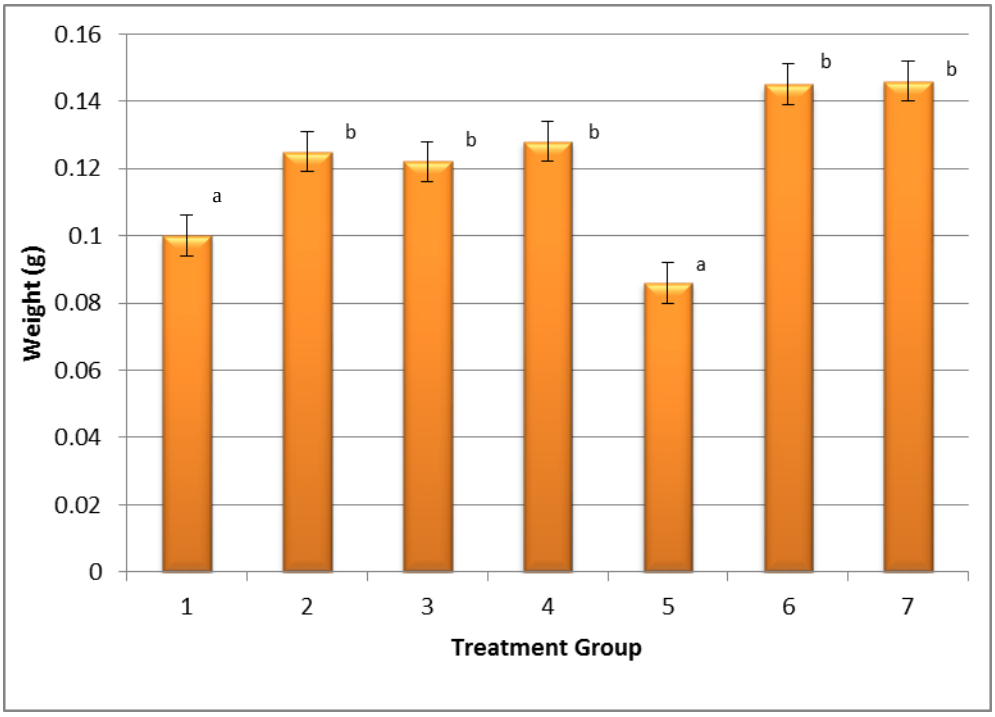

Figure 3.2. Average testis weight (+/-SE) for treatment group 1 (oral gavage), 2 (nasal lavage), 3 (live rSC54 subcutaneous injection), 4 (dead rSC54 subcutaneous injection, positive control group 5 (intramuscular injection of purified rmGnRH protein, 6 (oral gavage of non-modified SC54), and 7 (no treatment control). Oral gavage group 1 and positive control group 5 were similar with decreased testis weight when compared to control group 6 or non-treated animals in group 7. Values with different superscripts (a and b) are different ($P<0.05$; n=9).

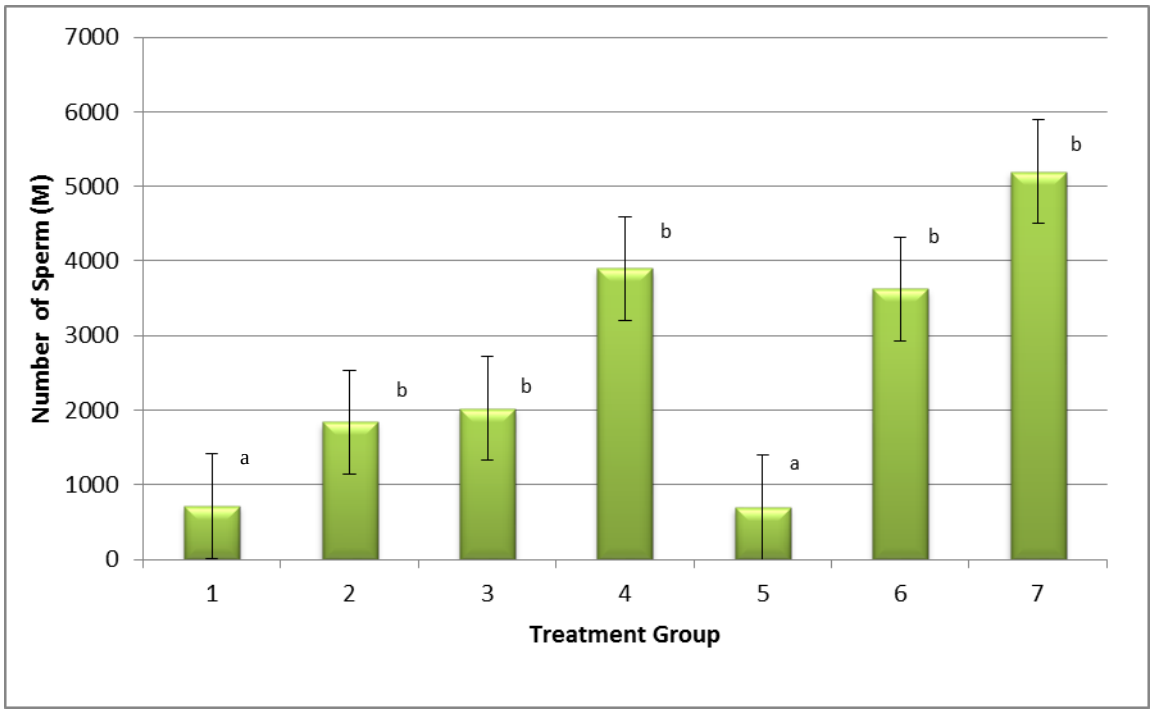

Figure 3.3. Average number of sperm (10^6) (+/-SE) for treatment group 1 (oral gavage), 2 (nasal lavage), 3 (live rSC54 subcutaneous injection), 4 (dead rSC54 subcutaneous injection, positive control group 5 (intramuscular injection of purified rmGnRH protein, 6 (oral gavage of non-modified SC54), and 7 (no treatment control). Oral gavage group 1 and positive control group (IM) injection 5 were similar with decreased sperm concentration when compared to control group 6 or non-treated animals in group 7. Values with different superscripts (a and b) are different (P<0.05; n=9)

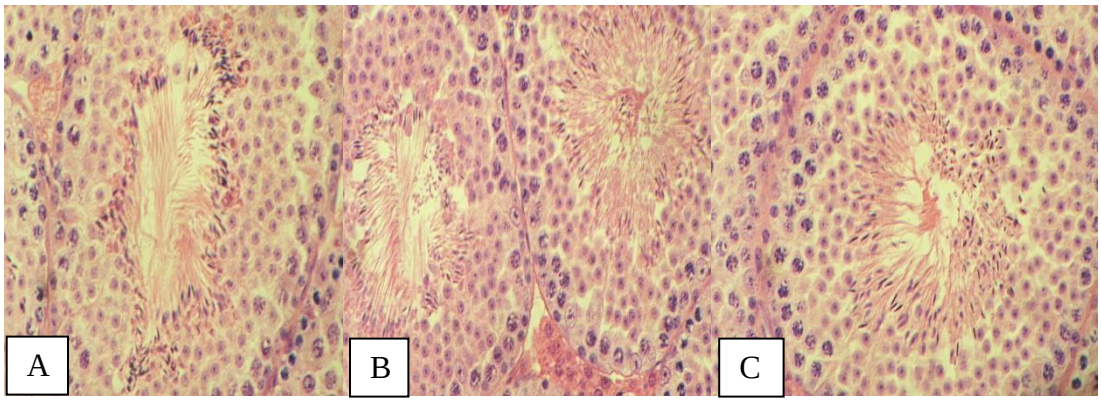

Figure 3.4. Histological slide preparations of mouse testes from oral gavage (A), non-treated control group (B), and positive control group (IM injection; C). Tissue was fixed in Bouin's solution for two days, washed twice and stored in 70% ethanol. Tissue was then mounted on paraffin blocks and 6µm sections then placed onto glass slides. The tissues were then stained with H&E Stain.

Chapter IV

FOAM PRESERVATION OF SC54

4.1 Introduction

Successful vaccines rely heavily on both a potent biological immunogen and also a delivery vehicle that protects that immunogen from environmental exposure. This is especially true for wildlife that might be exposed to harsh conditions for extended periods of time. The oral rabies vaccine (ORV), is packaged into biodegradable sachet containing liquid medium containing the Raboral V-RG™ vaccinia virus, that in turn is housed within a solid fish-meal polymer bait (Merial Inc.,United States, USA). This delivery system has been used extensively to orally immunize raccoons in the United States (Dietzschold et al., 2003). In recent studies, using raccoons housed in outdoor pens where they could be observed, the animals were given the ORV vaccine and were observed eating only the fish-meal polymer casing, and for others when the sachet was successfully punctured a significant portion of the vaccine was lost, while the animals manipulated the bait (Rosette et al., 2008). Animals that ate only the fish meal bait never developed immunity against rabies. But, for animals that ingested only a partial sachet, some actually ingested a sufficient amount of the immunizing virus to develop immunity, since a certain amount of vaccine is expected to be lost. Dietzschold et al. (2004) found that virus preservation and infectivity dropped by 10-fold after 1 week at 25°C or after 4 days at 37°C, resulting in insufficient effective V-RG to immunize the animals. Therefore, this loss of vaccine strength over time can be a major

concern for wildlife vaccines, especially since the majority of oral rabies vaccine baits are stored and deployed in the field for extended time periods. This could result in an ineffective vaccine dose being delivered to the animals in the wild.

Early work in preserving biological materials at high temperatures, showed that viscous liquids containing sugars or amino acids could be dried in a solid foam if a vacuum was applied (Annear, 1956). He used this process to preserve several types of bacteria in the dry state, for prolonged durations. In 1997, it was reported that biological macromolecules could be effectively stabilized by "foam drying" and that this process permitted of biological material to be stabilized with minimal loss of activity during the drying process or after storage (Bronshtein, 1997). The foaming technique has been used to stabilize herpesviruses, paramyxoviruses, flaviviruses, paroviruses, and retroviruses and hold them at room temperature or higher temperatures without significant loss of activity for up to 30 to 60 days (Alcock et al., 2010; House and Mariner, 1996; Pisal et al., 2006).

Freeze-drying, or lyophilization, has also been used to store biologicals in a dry state. However, this process has a number of drawbacks since freezing and subsequent equilibration at moderately low temperatures may damage labile biologicals, the process can take several days, and the lyophilized materials often must be stored at refrigerated temperatures to ensure stability. However, several of these problems can be ameliorated if the biological is preserved in dry foam, also called thermalstabilization. During the foamation process a liquid is transformed into a highly immobile, noncrystalline, amorphous state; a glass. This glass occurs from the ability of some sugars, such as sucrose and trehalose, to form glass-like matrices when dried and this resulting 'anhydrobiosis' allows cells, cellular machinery, proteins, and viruses to be essentially

arrested or frozen in the glass, allowing for long-term survival of an organism under conditions of extreme dryness (Abdul-Fattah et al., 2007; Bronshtein et al., 1997). Preserving an antigenic protein under anhydrobiotic conditions would permit the virus to remain intact for long time periods at relatively high (25-45°C) temperatures (Edwards and Slater, 2008).

The physical state of the glass produced during the foamation process has three major advantages for preserving biological compounds to liquid preservation. First, using foamation, a live bacterial vector would be more stable both during storage and when it is distributed to the field compared than if it was in the liquid form. Second, a dry material can more easily be incorporated into a solid bait matrix, thereby ensuring delivery of the entire vaccine dose. Finally, a greater volume or concentration of dry bacteria could be incorporated into bait, which may facilitate the delivery of a sufficient concentration of the vaccine to animals.

The process of foamation requires equipment that allows one to gradually decrease the pressure the sample is exposed to over time, reaching a low pressure of 50mTorr, while controlling the temperature of the sample. All of these factors affect the characteristics of the glass matrix formation. Conditions that lead to a higher glass transition temperature (T_g) are theorized to provide better protection for the sample. At temperatures above the T_g, the glass structure becomes disrupted and the preservation and stability of the substances in the matrix fails. The T_g of a glass matrix can be measured by differential scanning calorimety (DSC) which measures the change in temperature of a sample over time with a constant addition of thermal energy. The temperature at which the change occurs is the T_g (Bronshtein, 2004), and is theoretically the point at which a live organism would be lost, due to heat degradation.

This experiment describes how foamation can thermally protect both SC54 and modified SC54. It also serves as a proof-of-concept for the potential development of oral vaccines utilizing foamation to stablize live biologicals. Parameters tested in this experiment included the effect of different sugar formulation matrices and elevated temperatures for up to 21 days. Overall survivability was determined though observation of general death rates of bacteria after stabilization, over a 3 week time period.

4.2 Materials and Methods

4.2.1 Foamation Equipment and Procedure

Custom made equipment was used to make the foam products used in these experiments The apparatus includes a stainless-steal 1.5ft^3 chamber where the temperature and vacuum could be digitally controlled and monitored over the course of the process. Two lidded 96 well microtiter plates, containing the samples to be held at 25°C and 37°C over time, were placed in the foamation chamber that had been precooled to 10°C and equilibrated for 10 minutes. The chamber was maintained at 10°C and 1600 mTorr for 7 hours, or until the samples stopped bubbling. After the 7hr stabilization phase, the pressure was gradually decreased to 50 mTorr, over 2 hours, and the temperature was gradually increased to 50°C, over 2-3 hours, this temperature was held for an addition 10 hours. The plates were then removed and individually sealed in vacuum bags and one placed at 25°C and one at 37°C. A duplicate plate of non-foamed samples were also prepared and stored in vacuum sealed bags at either 25°C or 37°C. In total, there were four plates prepared, two foamed and two non-foamed, and one plate of each treatment stored at 25° or 37°C.

4.2.2 Matrix Solution Preparation

Sucrose and trehalose solutions were made at 100% w/v in PBS. The sugar solutions were heated as necessary to get the sugars to completely dissolve. The two sugar combinations used for testing are 80% Sucrose/ 20% Trehalose (80/20) and 50% Trehalose/ 50% Sucrose (50/50). After the sugars were dissolved the solutions were filtered through a 0.22 µm filter.

4.2.3 Preparation of Mixtures for Foamation

Two separate 100ml bacterial cultures containing 7 X10 ^{10} CFU/ml of modified SC-54 and 3.4X10 ^{10} CFU/ml of non-modified SC54 were centrifuged at 4000rpm for 15min and supernantant discarded. The cell pellets were resuspended with 20mls of PBS and centrifuged at 4000rpm. The supernatent was discarded and the bacteria were resuspended in 100mls of PBS. Ten microliters (10µl) of each culture was added to each of the 96 wells containing the different sugar combination treatments.

4.2.4 Plate Design

A 96-well microtitier plate was used for sample preparations. An individual plate was prepared to be held at either 25°C or 37°C. Location A refers to the wells that were used for analysis on each particular day during the experiment (Day 1,7, 14, and 21).

4.2.5 Twenty-one day time course survival:

Two different sugar combinations were used during this study to determine if the ratio of sucrose-to-trehalose in the foam has an impact on the survival of bacteria after being stabilized. Two sugar combinations, 80% sucrose/ 20% trehalose (80/20) and 50% trehalose/ 50% sucrose (50/50) were tested. After the stabilization process, the microtiter plates containing foamed and non-foamed bacteria were placed in vacuum sealed bags

and placed in temperature controlled incubators at either, 25°C and 37°C. On day 1,7,14, and 21, the sealed bags were removed from the incubators, punctured, samples removed, and the bags re-sealed, and returned to the storage temperature. On each designated day, foamed samples in location 2 on each plate were re-hydrated using 50µl of sterile water. Both the non-foamed and rehydrated foamed samples were transferred to a new 96 well microtiter plate containing 50µl of phosphate buffered saline (PBS), and each sample serially diluted by adding 10µl of each previous well to 90µl of PBS until reaching the end of the microtiter plate ending at a 10^{-10} dilution. A 10µl aliquot from each well was then plated onto duplicate LB Agar plates. The modifed SC54 samples were plated in duplicate on LB Agar plates containing kanamycin to maintain selectivity. The plates were incubated over-night at 37°C and the number of bacterial colonies on each plate recorded the next day. For example, if 5 colonies were present on the 10^{-6} dilution, the corresponding well from the processed samples at that time point contained $5x10^{6}$ CFU/ml. Survivability of bacteria was determined by evaluating the differences in number of bacterial colonies on each plate and at each dilution over 21 days. If foamation protected the viablity of the baceria, the number of bacetria should remain high over 21 days, as opposed to non-foamed baceria that are not protected.

4.2.6 Differential Scanning Calorimetry

The glass transition temperature (T_g) was measured by modulated DSC using a TA Instruments DSC 2920 (New Castle, DE, USA). Samples for DSC testing were prepared using the same carbohydrate matrices, but without bacteria and were foamed during the same process as the samples containing bacteria. Foamed samples were crushed to powder and loaded into hermetic aluminum crucibles (TA Instruments, New Castle, DE, USA) for evaluation. An empty control crucible, and one with crushed sample were

loaded into the DSC instrument, which was programmed to equilibrate the samples at 5°C, and then increase the temperature to 80°C at 2.5°C/5 minutes. The difference in the amount of heat required to increase the temperature of a sample and reference, is measured as a function of temperature. The heat capacity difference between the two samples reflects the glass transition temperature of the sample. Any change in heat capacity is observed as a change in the stability of the matrix and therefore is treated as the point at which the sample would no longer to viable.

4.3 Statistical Analysis

Data for the bacteria survival experiments were analyzed by an analysis of variance (ANOVA; SAS Institute Inc., 2011). Means were separated by the Student-Newman Keuls (SNK) mean separation technique (SAS Institute Inc., 2011).

4.4 Results

4.4.1 Survival of Bacteria

Survival of both bacterial strains was determined by recording the number of colony forming units at the lowest dilution that showed growth. The non-foamed and foamed CFU values were summed for each day and plotted over the 21 day time interval. Bacteria that were preserved by foamation survived longer than control bacteria. By day 7, no bacterial growth was recorded in any of the control samples, showing that these bacteria did not survive if they were not stabilized. However, bacteria stabilized by foamation, showed CFU values consistently in the 10^{-8} dilution through 21 days of storage (Figure 4.1). The initial CFU/ml of both bacterial samples (SC54 and rSC54) were $7.0x10^{9}$ CFU/ml. A substantial amount of bacterial loss is observed during the foamation process. However, once a foam has been produced and the matrix is stabilized, very little degradation and bacterial viability is lost.

4.4.2 Bacterial Type Sensitivity to Foamation

The foamation process maintained high bacterial survival of both types of bacteria. After Day 1 a substantial decrease in cell concentration occurs. However, beyond 7 days the concentration of each type of bacteria remains similar. (see Figure 4.2).

4.4.3 Effect of Temperature on Foamed modified SC54

Exposure of bacteria to 37°C after foamation did not negatively impact the survivability of bacteria, and high CFU values were maintained at both temperatures over the 21 day period (see Figure 4.3).

4.4.4 Sugar Composition and Effect on Survivability

The two different sugar compositions were equally efficient in stabilizing the bacteria ($P>0.05$). After the foamation process an immediate decrease in cell

concentration is observed, however, the cell concentration remains high between the two combinations of sugar (see Figure 4.4).

4.4.5 Differential Scanning Calorimetry

The glass transition temperature (T_g) of the 80:20 sugar combination matrix was 46.7°C (see Figure 4.5a), while the 50:50 combination transition temperature was 40.2°C (see Figure 4.5b). The glass transition temperature of each sample corresponds to the temperature where the bacteria will not remain viable. From these results, the 80:20 sugar combination provides thermal stability to a higher temperature than the 50:50 sugar combination.

4.5 Discussion

A method of thermally stabilizing live biologicals in a dry state may provide a valuable tool for improving oral delivery of vaccines to wildlife. One advantage of foamation is that it may eliminate the need to refrigerate live biological materials. Most live vaccines require low temperatures for long-term stability, however, if little degradation occurs at higher temperatures when the material is stabilized, then refrigeration would not be necessary, which would make it easier to store and transport sensitive biologicals and increase their effective life-span during dissemination in the field (Bronshtein, 1997).

These experiments demonstrate that foamation of modified SC54 protects the viability of bacteria at 37°C for 21 days. This temperature is similar to that likely to be encountered by oral vaccines placed outdoors. Therefore, foamation may improve the stability of vaccines used in baits increase the number of viable in each ‘dose’ of bait. However, in a recent study, mice treated orally with a foam dried formulation of attenuated *S.typhimurium* were not protected from infectious challenge, while bacteria

from a liquid culture induced protection (Edwards and Slater, 2008). This suggests that either the attenuation of bacteria decreases the ability of bacteria to withstand the foamation process, or the process itself causes a decrease in immunogenicity of live bacterial vaccines. This needs to be investigated further to determine if bacteria preserved by foamation exhibit similar properties.

4.6 Conclusion

The foamation process described by Bronshtein (1997) was applied to *Salmonella choleraesuis* strain 54 (SC54) and a recombinant version of SC54 (rSC54) that expresses a heterologous GnRH fusion protein. Various parameters that can affect over-all survivability of an oral vaccine were tested including exposure to elevated temperatures over long periods of time and differences in the foam matrix formulation to determine if the viability of a vaccine could be extended by incorporating dry, live biologicals into a completely solid bait matrix, and ensuring a potent dose of vaccine is orally delivered. Bacteria preserved in foam were thermally protected, therefore, it is possible that oral vaccines could be preserved using this technique.

Figure 4.1

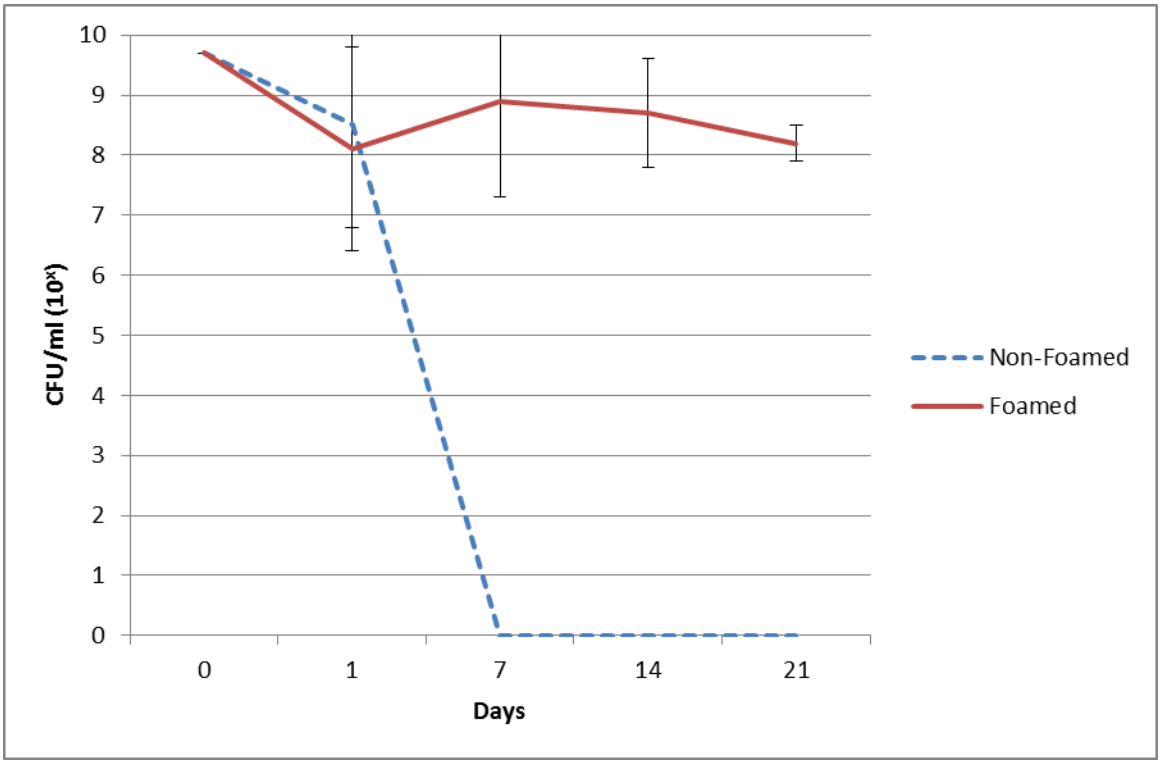

Figure 4.1. Survivability of live bacteria (+/-SE) in liquid sugar solutions (dashed line) or in a foamed sugar matrix (solid line) for 21 days. No bacterial growth was detected in non-foamed samples after Day 1. Bacterial colonies were counted from plate serial dilutions and values collected from the plates inoculated with the lowest bacterial dilution showing growth (n=8).

Figure 4.2

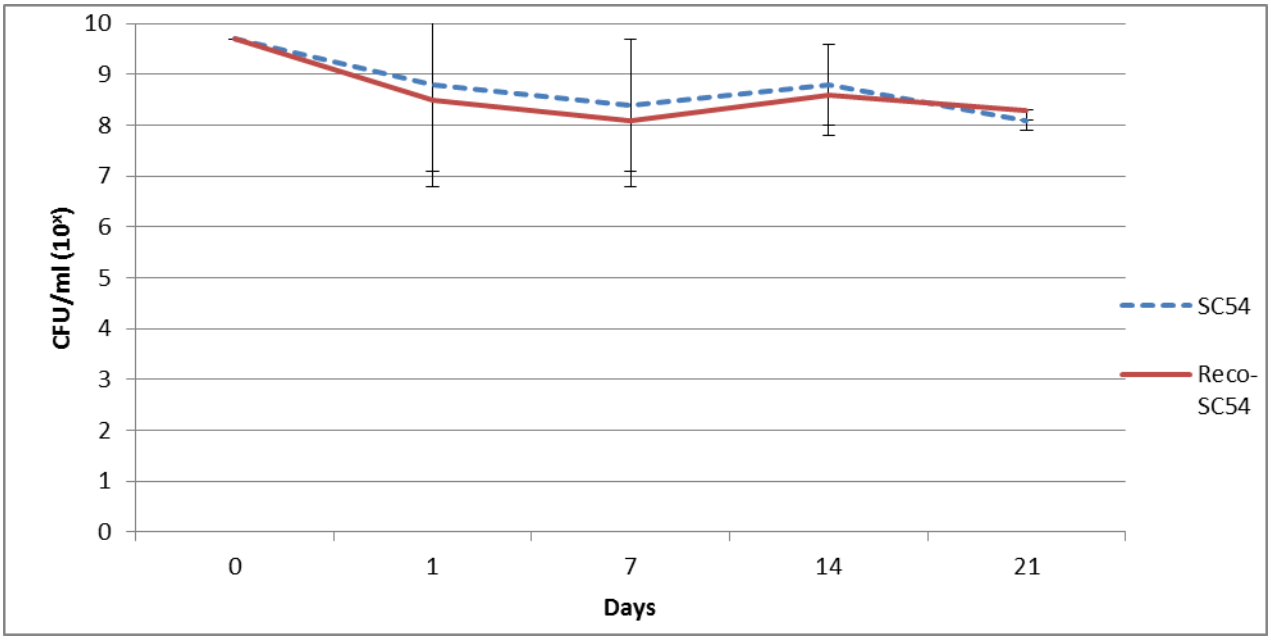

Figure 4.2. Sensitivity of recombinant and non-recombinant bacteria to the foamation process (+/- SE). Non-modified SC54 (dashed line) showed no apparent increased protection or resistance to the foamation process when compared to rSC54 (solid line) (n=8).

Figure 4.3

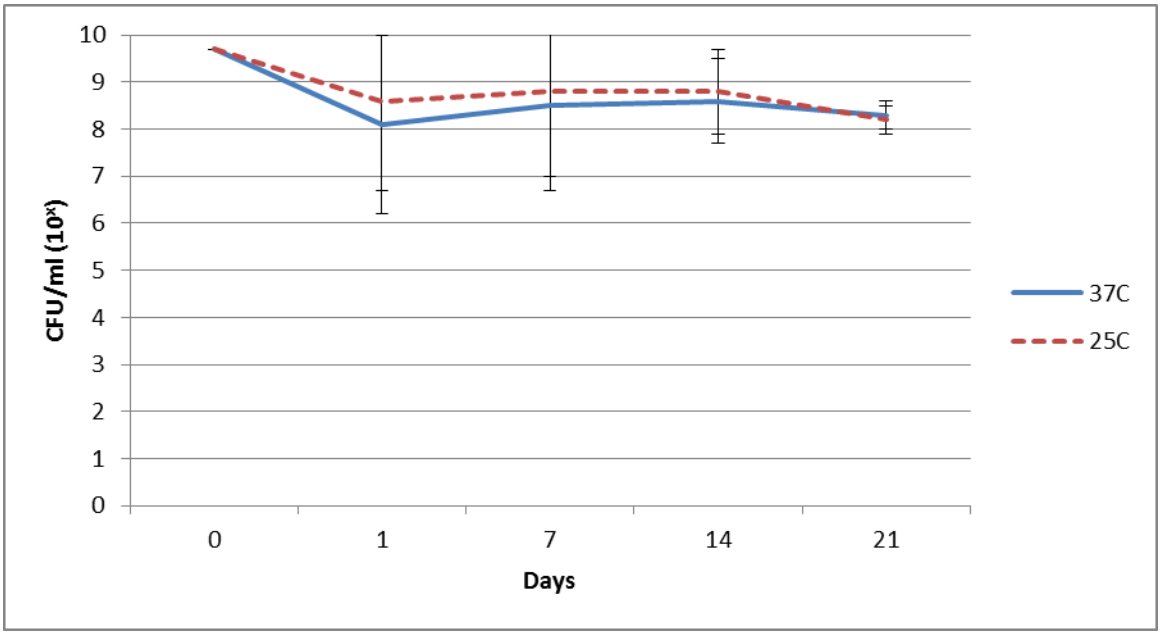

Figure 4.3. The effect of storage temperature and the survivability of foamed bacteria. Foamed samples were stored at 25°C (dashed line) and 37°C (solid line) for 21 days. An immediate loss of bacterial survival was observed from both temperature conditions after the foamation process. Samples stored at 25°C were generally protected better than those at 37°C (n=4).

Figure 4.4

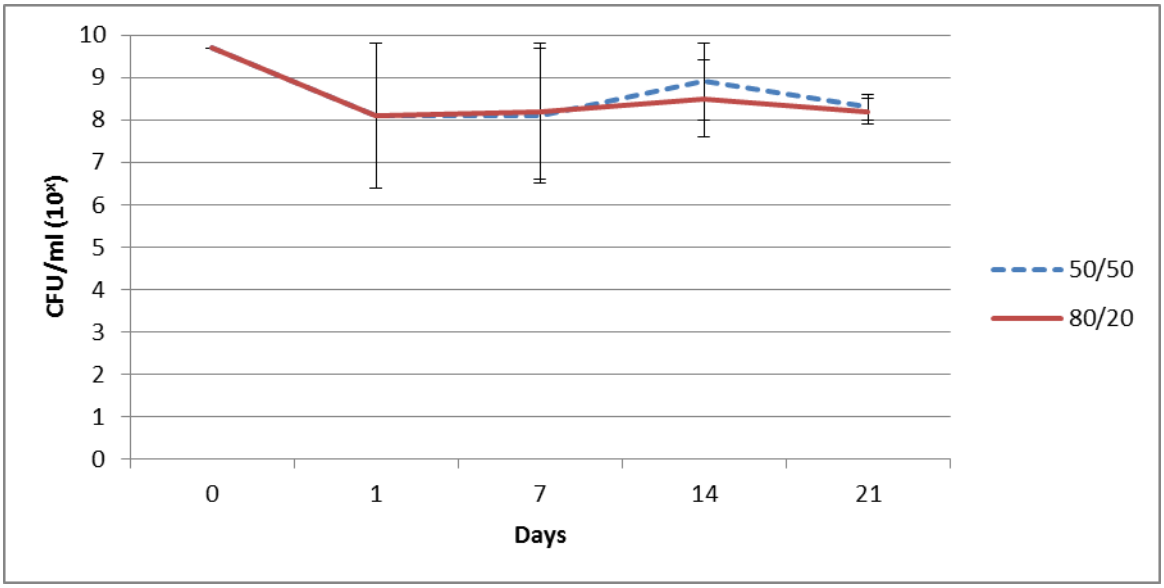

Figure 4.4. Two different sugar combinations and their effect on bacterial survivability. The two sugar combinations consisted of 50% Sucrose /50% Trehalose; dashed line, and 80% Sucrose/20% Trehalose; solid line) were used as matrix compositions for the foamation process. Both combinations were shown to be protective for the bacteria (+/- SE), however, a rapid loss of viable bacteria was experienced after the foamation process in both sugar combination samples (n=2).

Figure 4.5a

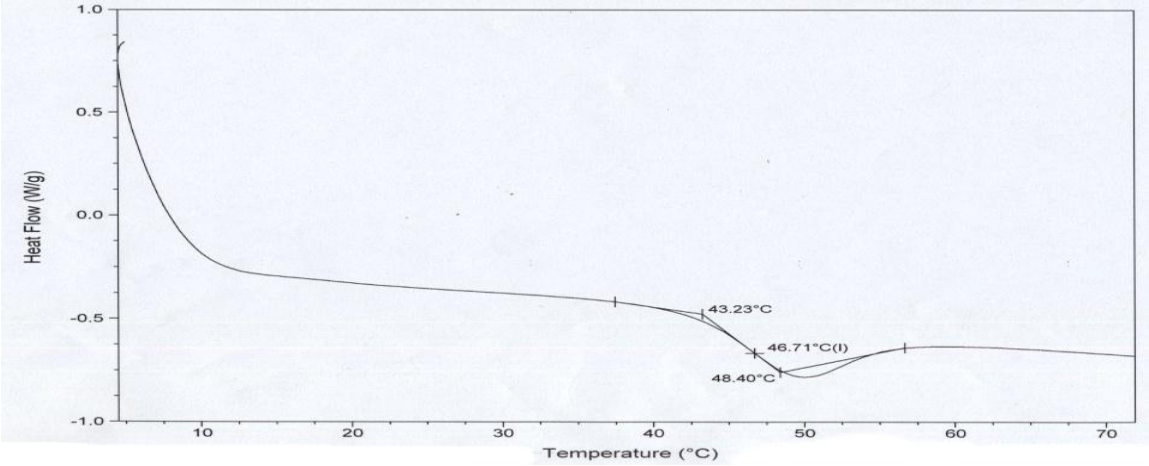

Figure 4.5b

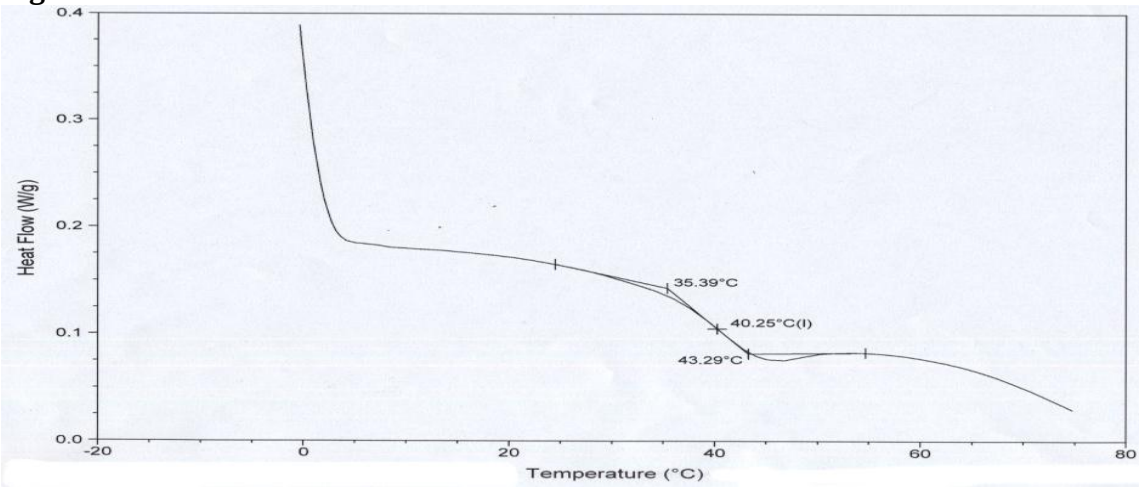

Figure 4.5 a and b. Differential Scanning Calorimetry (DSC) thermograms of foamed matrices composed of (a) 80% Sucrose and 20% Trehalose; and (b) 50% Sucrose and (50%) Trehalose. Glass transition temperature (Tg) was performed and calculated using a TA Instruments DSC 2920 (New Castle DE, USA). The glass transition temperature (Tg) of 50/50 sugar matrix to be 40.25°C and the 80/20 matrix was 46.71°C.

Chapter V

SUMMARY AND CONCLUSIONS

The ability to develop oral vaccines against infectious disease or as a means to contracept over-abundant wildlife species that serve as reservoirs of these diseases is well recognized. These oral vaccines can be delivered in bait form, which can be left at feeding stations the animals come to, rather than attempting to capture the animals (Cross et al., 2007). Oral vaccines have been deployed to vaccinate raccoons against rabies and this has encouraged research into other oral vaccines as a practical means to control other wildlife diseases including bovine tuberculosis (Aldwell et al., 1995a; Aldwell et al., 1995b; Buddle et al., 1995), brucellosis (Cheville et al., 1996; Stevens et al., 1996), and plague (Creekmore et al., 2002; Mencher et al., 2004; Rocke et al., 2004). However, successful delivery of oral vaccines depends upon the immunogen itself to elicit a protective immune response (Fooks, 2000). The success of the oral rabies vaccine program has relied heavily on the invasive nature of the vaccinia virus to stimulate both a systemic and mucosal immune response (Weyer et al., 2009). Similarly, enterobacteria that colonize within the host induce a strong immune response and can serve as potential carriers of heterologous antigens (Curtiss III, 2002). However, these organisms must be attenuated before they are used in a vaccine, so they will not cause the disease normally associated with their exposure. In addition, attenuation might also control secondary target species pathogenicity (Dougan et al., 1987). However, the use of genetically

modified bacteria as vaccine vectors carry the inherent risk of introducing a strain of bacteria in the environment that can possibly revert to the virulent state or by additional mutagenesis causing resistance to antibiotics. Reversion to a virulent or infectious state is the main social concern and environmental risk to using attenuated bacteria in vaccines.

Introducing a contraceptive antigen into a live, self-replicative vector to manage wildlife animal numbers is also politically sensitive. Even so, recent studies by the Centers for Disease Control and Prevention, have incorporated GnRH genetic sequences into the vaccinia virus to determine if it could be used to manage the growth of over-abundant disease carrying animals (Wu et al., 2009). In addition, a potential dual vaccine could protect against rabies and provide immunocontraception.

Of equal importance is the device needed to deliver the vaccine. This device must provide a stable, viable immunogen. A key feature for using a thermally protective matrix to maintain live immunogens is that they could ensure sufficient quantities of immunogen is available to mucosal inductive sites (Cross et al., 2007). Most oral vaccines currently utilize a liquid medium to stabilize the live organism. Changing from a liquid oral vaccine to a solid dose using foamation may improve the chance that a complete immunizing dose of the vaccine is delivered, since biological stabilized in foam remain potent for longer time periods. The foamation process also protects the live immunogen against thermal degradation if it exposed to elevated temperatures.

In these studies, I hypothesized that utilizing a *Salmonella choleraesuis* strain that is effective as an oral immune stimulant, immunoneutralization could be observed if the bacterium were made to express a immunogenic GnRH –fusion protein (House et al., 2001). I have demonstrated that a multimeric GnRH fusion protein can be stably expressed from *Salmonella choleraesuis* strain 54 by using pNS2TrcD as an expression

plasmid containing the rmGnRH transgene sequence. The modified SC54 was delivered to mice targeting the mucosal inductive sites via oral gavage and nasal lavage, as well as subcutaneous injection, for comparison. A measureable immune response was observed from mice treated by oral gavage indicating that the GnRH fusion protein was expressed at concentrations sufficient to induce an immune response. Additionally, a stabilization study was performed, using non-modified and modified SC54, to determine if foamation could protect live bacterium from elevated temperatures (Bronshtein, 2003). Samples that had been stabilized by foamation remained viable for 21 days. By combining these two technologies, an oral vaccine with a relative long shelf life against a variety of infectious diseases could potentially be developed.

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