IMMUNOCONTRACEPTIVE VACCINES AGAINST BRUCELLOSIS AND POPULATION GROWTH IN FERAL SWINE

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GARRETT PAUL SMITH

Abstract

Feral swine are a nuisance species across the United States that costs around $1.5 billion each year in agricultural, environmental, and personal property damages. In the last ten years the population of feral swine is estimated to have quadrupled and novel population control methods are needed. Furthermore, feral swine are known carriers of zoonotic diseases such as brucellosis, which threatens both livestock biosecurity and public health. Recombinant multimeric gonadotropin-releasing hormone (mGnRH) has been previously used as a subunit vaccine to induce immunocontraception in feral pigs. However, potent adjuvants and large amounts of purified antigen are needed to elicit a robust anti-GnRH immune response and current delivery methods are limited. *Brucella suis* strain VTRS2 can be used as a novel platform to deliver mGnRH without the use of antibiotic resistant markers. Strain VTRS2 was created by deletion of the LPS biosynthesis gene *wbo*A as well as the *leu*B gene required for leucine biosynthesis inside the nutrient-depleted intracellular environment occupied by *Brucella*. Mutations in *wbo*A are known to attenuate *Brucella* strains such as the vaccine strain *B. abortus* RB51, however strain RB51 is rifampin resistant and has poor efficacy in swine. Strain VTRS2 confers significant protection against *B. suis* challenge in mice and additionally shows evidence of protection in feral swine. Furthermore, the mGnRH antigen can be delivered using the pNS4 plasmid (which expresses *leuB* under its native promoter) thus maintaining the plasmid in strain VTRS2 under leucine-deficient conditions while expressing recombinant antigen in the host. The

murine model was used to determine the clearance kinetics of strain VTRS2-mGnRH and to measure vaccine efficacy against challenge by virulent *B. suis* 1330. Subsequently the effects of the VTRS2-mGnRH vaccine on fertility were assessed in breeding trials in mice. Strains VTRS2 and VTRS2-mGnRH were found to be protective against virulent *Brucella suis* challenge. Strain VTRS2-mGnRH elicited an anti-mGnRH antibody response in vaccinated mice, though an effect on fertility was not observed. An improved vaccine against brucellosis in swine, which also confers immunocontraception without the introduction of antibiotic resistance, could become an important tool in the management of this nuisance invasive species.

Public Abstract

Feral swine (*Sus scrofa*) are a major invasive species in the United States. Their population is estimated to have quadrupled in the past ten years and their geographic range has expanded to include at least 39 states. In addition to causing over $1.5 billion annually in agricultural, environmental, and property damages, feral swine also carry several diseases of public health and agricultural significance including influenza, leptospirosis, and brucellosis. Among these diseases, brucellosis, caused by the bacterial organism *Brucella suis*, is of particular concern because of its ability to cause reproductive losses in domestic swine and cattle as well as a debilitating febrile illness in humans. The disease has been eradicated from domestic livestock in the United States, and reintroduction could have severe consequences for both animal agriculture and public health. With the continued expansion of the feral swine population, the potential for spillover of disease into domestic livestock and humans increases. Additional tools are therefore needed to aid in both population and disease control. Immunocontraceptive vaccines have previously been used to reduce population growth in wildlife, and have been proposed for use in feral swine. Immunocontraceptives work by introducing an immunogenic form of a reproductive hormone which then causes an autoimmune response against the natural form of the hormone produced by the animal. This work describes the development of the *B. suis* vaccine strain VTRS2-mGnRHb, which was created from a virulent strain of *B. suis* to make an attenuated live vaccine capable of delivering the immunocontraceptive antigen mGnRH. The goals of strain VTRS2 were to act as a vaccine which protect against virulent *B. suis* challenge and which confers an infertility effect in the mouse model. An improved vaccine against brucellosis in feral swine, which also confers an infertility effect, could become an important tool in the management of this nuisance invasive species.

Dedication

This dissertation is dedicated to my family whose love and support has never wavered. Especially to my wife and daughter who remind me every day that there is nothing more precious than the love that lets us share our name.

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Garrett Smith

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CHAPTER 1: LITERATURE REVIEW

Brucella and Brucellosis: Immune response and current approved vaccines

History and taxonomy of Brucella:

Brucellosis is one of the oldest known zoonotic diseases, with descriptions matching that of the disease dating back to those of a febrile illness associated with eating dairy products in the Roman Empire (1, 2). One of the causative agents of the disease, *Brucella melitensis*, was first discovered in 1887 by Sir David Bruce and isolated from a soldier stationed in Malta who succumbed to the disease; however, the zoonotic nature of brucellosis was not discovered until 1905 when the same organism was isolated from goat cheese (1, 3). Since the initial discovery of *B. melitensis*, many other species of distinctly related *Brucella* have been identified. *B. abortus* and *B. suis* were the next to be discovered (1897 and 1914, respectively). *B. melitensis*, *B. suis and B. abortus* are considered the most clinically significant species in order of their virulence (4). While it has been debated whether all of the nomenspecies of *Brucella* are in fact distinctive species rather than subspecies of *B. melitensis*, the *Brucellae* continue to be regarded as separate species due to their notable host-species preference in animals (5, 6). *B. melitensis* demonstrates a host predilection for sheep and goats, *B. abortus* for cattle, and *B. suis* for swine. There is significant overlap among the host ranges of each species, however, and each of these three classical species of *Brucella* can cause a similar abortifacient disease in multiple hosts. For example, *B. suis* is known to become established in populations of cattle, and *B. abortus* has been isolated from feral swine (5, 7).

In addition to the three classical species of *Brucella*, other species with specific host preferences are continually being identified. Some of the more well-known species infect domestic animals such as *B. ovis* and *B. canis,* which primarily cause epididymitis in rams and dogs, respectively. Other species are known only to infect wildlife reservoirs. The earliest known of these species, *B. neotomae,* is of low-pathogenicity and was isolated from desert wood rats (*Neotoma lepida*) of the Southwest United States in 1957 (8, 9). *B. ceti* and *B. pinnpedialis* are species known to infect and cause disease in marine mammals. These were first described in 1994 and appear to cause a similar reproductive disease in marine species to that seen in land mammals (4, 10, 11). Most recently, several named and unnamed *Brucella* species have been identified in a variety of host species, most of which have yet to be fully characterized. Among them are *B. microti* which has been isolated from voles (*Microtus arvalis*), red foxes (*Vulpes vulpes*), wild boars (*sus scrofa*), and soil samples, and *B. inopinata* which was isolated from a human breast implant infection (12-15). *B. microti* is particularly interesting as it appears to cause a lethal infection in experimentally infected mice, which is otherwise uncommon (13). Other potential novel species have been isolated from red foxes, Nile catfish, baboons, and frogs (16-19). The demonstration of the ability of *Brucella* to establish infection in fish and frogs is an interesting one, as it is a significant deviation from the perception that brucellosis is limited to mammals (20, 21). It is not currently known which of these species are in fact capable of causing disease in humans or domestic mammals, nor how closely related they are to the classically recognized species of *Brucella*. The new species, however, do appear to be distinct both genetically and phenotypically by the conventional tests for differentiation of *Brucellae* (6). They also appear to be distinctly different from *Ochrobactrum* species, the closest relatives of *Brucella* (6, 22). With the exception of *B. inopinata,* these new *Brucella* species appear to be

emerging diseases of wildlife, along with the classically recognized species, that could potentially threaten both human and domestic animal health. Among the *Brucellae, B. suis* in particular is found in many wildlife species as diverse as bison (*Bison bison*), elk (*Cervus elaphus*), reindeer and caribou (*Rangifer tarandis*), foxes (*Vulpes vulpes*), wild hares (*Lepus capensis*), and especially feral swine (*Sus scrofa*) (1, 23, 24). Among these, feral swine are the most widespread and of the most concern for infection of humans or domestic animals. Human infections by *B. suis* from feral swine have been documented. The growth of feral swine populations in the United States presents a unique challenge to brucellosis control efforts (24-27). Worldwide, brucellosis is a re-emerging disease that remains poorly controlled (28).

Pathogenesis of Brucella infection:

Brucella spp. are fastidious non-motile Gram negative coccobacilli that act as facultative intracellular pathogens (29). Their intracellular niche aids in evasion of the host immune response and establishment of chronic infection in phagocytic cells such as macrophages. The preferred niche within the macrophage has led to the adaptation of several unique virulence factors that contribute to both cell invasion and immune-evasion upon entry into host cells (30).

Like many pathogenic bacterial species capable of systemic infection, *Brucella* spp. enter the host via crossing of the mucosal epithelium at the site of exposure and transport to local lymph nodes (30). Though the mechanism of initial entry is not fully understood for all routes of exposure, it is known that most *Brucella spp.* code for two ureases which aid survival in the acidic conditions of the stomach and upper intestinal tract and therefore facilitate entry via the oral route of exposure (31). Of the two ureases, one (UreI) is especially critical for this function as strain with mutations in the *ure1* operon attenuate both *B. suis* and *B. abortus* (32). Paixao et

al. (2009) showed that two other well-studied virulence factors, lipopolysaccharide (LPS) and the type-IV secretion system (T4SS), are also required for entry via oral exposure (33).

Either at the site of initial infection, during bacteremia, or in regional lymph nodes the bacteria enter into phagocytic cells. The route of entry is a major determinant of the outcome of infection and is most affected by the LPS (34). The LPS is one of the most studied and most important virulence factors of *Brucella*. Unlike other Gram-negative pathogens such as *Escherichia coli* and *Salmonella enterica*, the *Brucella* LPS is a poor activator of TLR4 signaling, an important component of innate immunity against bacterial infection. This adaptation serves to greatly reduce inflammatory reactions against *Brucella* invasion as compared to the severe inflammatory reactions associated with the LPS endotoxins of *E. coli* and *S. enterica* (35, 36). *Brucella* LPS is present on the bacterial outer membrane surface and consists of three major components, hydrophobic lipid A, a core oligosaccharide which is divided into inner and outer components, and the O-side chain polysaccharide (37). The lipid A serves as the attachment to the outer membrane. The core is highly conserved among LPS-containing organisms and is mostly comprised of neutral sugars, primarily glucose (37, 38). The O-side chain, or O-polysaccharide (O-PS) is the most variable component and is the structure responsible for the uniqueness of *Brucella* LPS (36, 37, 39). The O-PS consists of variable lengths of mannose-derived polymerized N-formylperosamine and in *Brucella* the average length of each polymerized molecule is more than double that of bacteria with toxic LPS (36, 39). The LPS of *Brucella* strains with a complete O-PS demonstrate reduced activation of complement, low toxicity to infected host cells, and resistance against anti-microbial host peptides as compared to other bacterial species as well as *Brucella* strains without an intact O-PS (37, 39). Strains with a complete O-PS are referred to as smooth strains, while those lacking the O-PS are

termed rough and are markedly attenuated (36). This distinction is an important component for understanding host-*Brucella* interactions, vaccination strategies, and key differences between both naturally occurring and laboratory-generated smooth and rough strains and species. Serological diagnosis based on antibodies against LPS is also the primary method of brucellosis testing and surveillance.

The efficiency of entry into host phagocytes and eventual access to the preferred intracellular niche of *Brucella* is largely dependent upon LPS structure. Rough strains enter macrophages more efficiently; however, this is primarily through phagocytosis. Bacteria that enter through this route are unable to replicate and the majority are quickly killed by the oxidative burst (34). The increased uptake is likely due to decreased role of the rough *Brucella* O-PS in evading TLR4 and other inflammatory signaling pathways. The decreased ability to replicate and establish infection is due to both increased inflammatory signaling in the phagocyte and failure by rough strains to acidify the phagosome, a required step for avoiding phagolysosomal fusion and respiratory burst following phagocytosis (30). Virulent smooth strains are able to enter host macrophages via a different mechanism in which the bacteria associate with cholesterol and glycosphingolipid-rich domains on the host cell surface and are co-internalized in lipid rafts (40). This method of entry is more akin to pinocytosis rather than the inflammatory process of phagocytosis (41). Once inside the host cell, smooth strains are able to evade phagolysosomal fusion via acidification of the *Brucella*-containing vacuole (BCV), an important early step in the establishment of intracellular infection (30). Smooth LPS is also implicated in resistance against oxidative damage, resistance against complement, and inhibition of apoptosis of infected cells (42).

Once virulent *Brucella* have avoided oxidative burst upon internalization into the macrophage, the bacteria then manipulate intracellular trafficking to maintain the BCV. These processes allow for replication of the bacteria and maintenance of intracellular infection by preventing apoptosis of the host cell. This is accomplished by manipulating the BCV to display cellular markers normally found on endoplasmic reticulum (ER) (43). The type-IV secretion system (T4SS), a complex protein apparatus which translocates recently-identified effector molecules inside the host cell, is implicated in this process. The translocated effector molecules re-direct intracellular-trafficking and disguise the BCV from receptors that would otherwise signal destruction of the infected cell via the apoptosis cascade mechanism (43). Assembly of the T4SS appears to be dependent upon the ability of smooth strains of *Brucella* to acidify the BCV (34).

In addition to *Brucella*, several pathogens of both plants and animals employ a T4SS to secrete effector molecules into host cells, including *Agrobacterium tumefaciens, Bordetella pertussis*, *Helicobacter pylori*, and *Legionella pneumophila* (44). These effector molecules play a role in the modification of host defenses, aid in the development of an intracellular growth environment, and induce metabolic changes in host cells to alter nutrient availability and improve the intracellular niche (45). The T4SS functions as a means of conjugative DNA transfer, contact-independent DNA transfer, or of the transfer of proteins into host cells (45).Transcription of the secretion system and its effector substrates is tightly regulated and often dependent upon environmental cues such as nutrient availability, pH, and quorum sensing signals (46, 47). The actual mechanisms by which the T4SS and its substrates affect the host are poorly characterized; however, progress towards increasing our understanding of these components in *Brucella* as well as other bacteria continues to be made since the first description of the apparatus

in the *virB* operon in a *Brucella* species in 1999 (48, 49). *VirB* mutants are attenuated and lack the ability to display ER markers on the BCV. Specific effector molecules of the T4SS in *Brucella* have only recently been identified, and of them only one, RicA, has a known function (43, 48, 50, 51).

As discussed in reference to the role of smooth LPS, the most critical component of establishing an intracellular infection is avoiding or surviving the oxidative burst. *Brucellae* have several virulence factors, in addition to LPS, that aid in avoiding innate immune responses. A newly characterized virulence factor, InvA, appears to downregulate the oxidative stress response signaling pathway (52). InvA belongs to a family of enzymes that degrade oligophosphate nucleotide stress signaling molecules inside the host cell and thereby reduce activation of the cellular pathways necessary for sensing and responding to the stress of bacterial invasion. Similar enzymes are found in other intracellular pathogens such as *Rickettsia prowazekii* and *Salmonella* Typhimurium (52). Another recently discovered system in *Brucella* to reduce immune-signaling involves the TIR-domain containing molecules BtpA and BtpB (53). TIR-domains (conserved TLR/IL-1R superfamily domains) are present on the mammalian immune signaling molecules toll-like receptors (TLRs) and interleukins and serve as the point of interaction for intracellular signaling upon TLR activation. In an act of molecular mimicry, some pathogens of plants and animals have adapted to express TIR-domains that serve to interfere with TLR signaling within the host-cell. These molecules have only recently been described in *Brucella* and are not yet fully understood, however BtpA has been shown to interrupt TLR-2 and TLR-4 signaling mainly via interaction with the downstream signaling molecule MyD88. This interaction decreases TLR signaling and reduces activation of the infected host cell in response to infection (54). BtpB has also been shown to have an anti-inflammatory effect *in vivo* in a

mouse model, and Btp mutants cause increased granuloma formation during the course of infection as compared to wild-type strains (53). It is not yet known if the molecules serve redundant or separate functions as they appear to have overlapping yet distinctly different effects on the host cell (53).

Another conserved bacterial virulence factor found in *Brucella spp.* is the family of periplasmic molecules known as cyclic β-1,2-glucans (CβG), though the CβG of *Brucella* appear to be regulated differently from those of other pathogens (39). CβG is required for intracellular survival and aids LPS in modification of lipid-rafts and preventing phago-lysosomal fusion thereby allowing the T4SS to interact with ER markers and establish the BCV (39, 55). In addition to reducing activation of oxidative pathways in the host, *Brucella* has also evolved mechanisms to resist oxidative damage. This includes the expression of the enzymes superoxide dismutase (SOD) and catalase, with SOD appearing to play a larger role in virulence (56, 57). SODs are metalloenzymes, among the most conserved enzymes in biology, that catalyze the conversion of the reactive oxygen intermediate (ROI) superoxide into oxygen and hydrogen peroxide (58). Catalases and other enzymes are able to degrade hydrogen peroxide to further reduce damaging ROIs in the cellular environment. In the *Brucellae*, two SODs exist, a cytoplasmic Fe-Mn dependent form which catabolizes ROI produced by bacterial metabolism and a periplasmic Cu-Zn dependent form. While both play a role, the Cu-Zn SOD (SODc) is most important for virulence and is encoded by the gene *sodC*. Upon translation, SODc is translocated to the periplasm where it is required to resist the respiratory burst (57, 59-61). SODc has also been used as a protective antigen both expressed homologously in attenuated strains of *Brucella*, and heterologously in *E. coli*. It has also been expressed in DNA vaccines with mixed results (62-65). Other less-studied genes involved in surviving oxidative damage include *norD*

nitric oxide reductase (66) and peroxiredoxin *ahpC* (67). Like catalase (encoded by *kat*E), *ahp*C is involved in detoxification of hydrogen peroxide to water and gaseous oxygen. The two genes appear to complement one another and although mutation in either one gene has little effect on *in vivo* attenuation in a mouse model, *ahp*C/*kat*E double mutants are highly attenuated (67).

After *Brucella* eludes the initial innate immune response, the bacteria become established in their intracellular niche and replicate within the BCV. Other adaptations that allow for survival inside the nutrient-limited environment inside the phagocytic cell include alternative pathways for biosynthesis of otherwise limited nutrients (68, 69). An abundance of pathways for *de novo* amino acid synthesis in particular have been described and *Brucella spp*. can survive using certain single amino acids or solely ammonium salts as the nitrogen source (68, 70-72). One amino acid for which synthesis can occur from nitrogenous precursors is leucine (68). Using a library of *Brucella abortus* genes in auxotrophic mutants of *E. coli* unable to grow without certain amino acids, Essenberg and Sharma (1992) identified an isopropyl malate dehydrogenase, LeuB, in *Brucella* that allowed for growth in the absence of leucine (72). *Brucella abortus* RB51 and *Brucella suis* deletion mutants in the *leuB* gene are unable to grow on minimal media deficient in leucine, and growth can be restored by complementation of *leuB* on a markerless plasmid (this work, (73)). This trait can be exploited for long-term maintenance of expression plasmids in *Brucella* candidate vaccines without the use of antibiotic resistance markers as first described by Rajasekaran et al. (2008) and expanded upon in this work.

Host immune response against Brucella infection:

Though humoral immunity does play a role, the dominant host immune response against *Brucella* infection is principally characterized as a Th1-dominant cell-mediated response with both innate and acquired components. While the response is dependent upon the strain, dose,

host susceptibility, and prior exposure or vaccination, it is well established that cell-mediated immunity is required to resist and clear infection (23, 74-77). *Brucella* antigens trigger a predominantly Th1 CD4+ response that is characteristic of immunity against intracellular pathogens (78). This response is triggered by bacterial antigen recognition, processing and presentation of 8-12aa antigen peptides on major histocompatibility complex class II (MHCII) molecules on the infected macrophage (78, 79). TLR signaling is involved in recognition of infection and *Brucella* infection has been shown to activate TLR-2, TLR-4 and TLR-9 (80, 81). The main mechanism of recognition of bacterial presence in the case of *Brucella* infection is by TLR-9 which has affinity for bacterial DNA and helps drive the Th1 response (82). In addition to classical TLR-mediated pattern recognition, three immunodominant MHCII-associated peptide antigens were recently identified by studying the IFNγ response of *B. melitensis* infected humans to different predicted peptide antigens. The three dominant antigens included a protease, a protein associated with the VirB T4SS, and a periplasmic protein of unknown function (83). MHCII antigen presentations along with increased interferon-gamma (IFNγ) production by the infected cell are principally responsible for activation of CD4+ T-lymphocytes which drive the Th1 response. This response consists of increased production of IFNγ by both macrophages and γδ and CD4+ T-cells. IFNγ is the hallmark of cell-mediated immunity and is considered the most important component of host resistance to *Brucella* infection. Increased IFNγ production has a direct inhibitory effect on *Brucella* (84), increases activation of macrophages (78, 85), and increases differentiation of CD4+ and CD8+ cytotoxic T-lymphocytes (86-88). Activated macrophages contribute to clearance of infection primarily by increasing production of ROI (87). CD8+ lymphocytes aid in increasing IFNγ as well as in destruction of the infected cell and elimination of the bacteria (86). Humans and animals deficient in IFNγ production appear more

susceptible to disease, and in humans a specific allele for IFNγ that correlates with decreased IFNγ production has been identified (77, 85). Another important component of CMI is the production of tumor necrosis factor-alpha (TNFα) by both infected macrophages and natural killer cells (NK). TNFα also activates macrophages and NK cells themselves which then act to both secrete IFNγ and TNFα as well as lyse infected cells (86, 89). During the course of infection, TNFα is increased however not to the relative degree of IFNγ (83, 86). This is associated with the ability of *Brucella* to downregulate TNFα production during the course of infection associated with the outer-membrane virulence factor, Omp25 (90). *Brucella* also appears to downregulate MHCI antigen presentation and the CD8+ cell response, further contributing to the ability of the bacteria to evade clearance and establish chronic infection (79). Other key cytokines associated with Th1 response that are increased during the course of *Brucella* infection include IL12, which induces T-cell differentiation and IFNγ production early in infection, and the proinflammatory cytokines IL1 and IL6 (78, 85, 86). Decreased production of these cytokines is also associated with establishment of chronic infection (91).

The Th2 cytokines IL4 and IL12 are not normally increased in infection although there is a Th2 humoral antibody response associated with infection with LPS being the dominant antigen (75, 78, 92, 93). However, the main antibody isotype detected is IgG2a which strongly correlates with Th1-type immunity as opposed to IgG1a which is IL4 mediated and associated with the Th2 response and IL10 inhibition of interferon production (89). IgG2a and IgG3 isotypes facilitate opsonization-mediated phagocytosis of the bacteria, an effective killing mechanism even against smooth strains of bacteria (30, 89). The limited role of Th2 responses in clearance of infection helps to explain why killed brucellosis vaccines provide less protection than live vaccine strains

which are internalized by host cells to induce Th1 responses (89, 94). A switch from Th1 to a Th2-dominant response is also associated with chronic infection in mice and humans (95, 96).

Complement appears to play a role in immunity but this role varies depending on the strain. Rough strains, lacking complement-resistant LPS, readily activate complement thus contributing to their attenuation whereas smooth strains are highly resistant (97, 98). However, in both cases, activation of complement is via the mannose-lectin binding and classical pathways, not the antibody-independent alternate pathway. Smooth bacteria are most likely to encounter classical, antibody-mediated complement killing although they are more affected early in infection when IgM predominates over IgG and before levels of IgG rise to peak concentrations (89, 97, 98).

Immunity against *Brucella* infection has been most-studied in macrophages and dendritic cells *in vitro* and in the mouse model, especially in the context of vaccines and correlation to human infection. While the mouse model is well-established for *in vivo* brucellosis research, some key differences appear to exist between mice and natural hosts such as pigs and cattle and between individual strains of mice (30, 54, 86, 95). Mice (*mus musculus*) are not a natural host of *Brucella* and are considered resistant to natural brucellosis infection, however the two most-studied mouse strains in brucellosis research, BALB/c and C57BL/6 differ in their susceptibility. This is likely attributed to the inability of BALB/c mice to maintain IFNγ levels after the initial infection, causing a more natural chronic infection in that strain. This makes BALB/c the recommended model for both human and natural host disease (95, 99). These differences also affect the presentation of the disease in different species, especially between mice, humans, and natural hosts of the infection. Mice for example rarely present with abortion, though the placenta does become colonized (95). In natural hosts of infection, however, abortion is the major clinical

indicator of disease. This may be due in part to the ability of the bacteria to down-regulate TNFα, IL12, and IL1 in placental trophoblasts early in infection followed by delayed up-regulation of the chemotactic proinflammatory cytokines IL8 and GCP-2 in bovine cells (100). The resulting granulocytic infiltration, related to IL8 up-regulation in the bovine, only reliably occurs in mice if they are infected mid-gestation (95, 100). Mice also do not shed the bacteria from venereal and placental secretions, a hallmark of the course of infection in the natural host. Humans, like mice, are not natural hosts of the disease. Unlike natural hosts, both humans and mice frequently experience splenomegaly associated with infection. Isolation of the organism from infected splenic tissue is an important component of assessing vaccine efficacy in the mouse model. Humans and mice both also show evidence of granulomatous inflammation in the reticuloendothelial organs. This inflammation is consistent with delayed-type hypersensitivity and is associated with chronic infection similar to that seen in cases of tuberculosis (30, 95, 101, 102). Unlike in humans, granulomatous lesions are seldom found outside the reticuloendothelial organs such as the spleen and liver in mice whereas lesions commonly occur in the brain, joints, and bone of human patients (95, 101). Despite these differences in host-pathogen interactions between the various host species, mice are the most practical brucellosis model given the constraints of working in a BSL-3 laboratory setting and continue to be a valuable and highly meaningful model for studying the disease. It is nevertheless important to consider that differences in the behavior of *Brucella spp.* in the different hosts cause different manifestations of the disease.

Brucellosis in humans:

Though humans are not natural hosts of *Brucella*, brucellosis is one of the most widespread zoonotic diseases in existence. Roughly 500,000 new cases are reported annually and

the true incidence is likely higher as the disease is easily misdiagnosed and underreported in developing countries (28, 103). Worldwide, the disease is mainly acquired from consumption of contaminated dairy products or through workplace exposure in places such as abattoirs or laboratories (3). Workplace exposure occurs especially through the aerosol route, as the bacteria readily aerosolize and an infectious dose can be as low as 10 or fewer organisms. This fact, combined with the debilitating nature of the disease, led to *B. suis* being the first organism weaponized by the United States Army. Moreover, *B. melitensis*, *B. suis*, and *B. abortus* are considered Category B Select Agents capable of use in bioterrorism by the CDC Bioterrorism Preparedness and Response Office (4, 104-106). Of the recognized species of *Brucella*, *B. melitensis* is considered the most pathogenic to humans and is the most common species implicated in infection in developing countries, followed closely by *B. suis* and *B. abortus*. *B. suis* is especially similar to *B. melitensis* in pathogenicity, and is the biggest concern in wildlife reservoirs (107). All of the species of *Brucella* have been known to cause zoonotic infections except for *B. neotomae*, *B. ovis*, and the recently identified *B. microti* (1, 27, 108, 109).

Clinically, the classical manifestation of brucellosis is a severe undulating fever which is often debilitating to the patient. The disease rarely results in death; more commonly symptoms recrudesce periodically as a chronic infection becomes established. Patients with chronic infection commonly experience severe arthropathies and neurological symptoms and the bacteria are very difficult to eliminate even with long-term antibiotic therapy (103). Though the disease is sexually transmitted in host-species, the bacteria do not localize to reproductive tissue in humans as readily as in animals. Human-to-human transmission has been reported, but is extremely rare, and the infection is not considered contagious in humans, who are regarded as dead-end hosts (1, 3, 110). Reproductive symptoms in humans are relatively uncommon although abortion has

occurred in pregnant women and some men have complained of prostatitis or orchitis. Instead, the presentation is typically more ignominious with “flu-like symptoms” being the most common complaint. This characteristic, combined with the variable incubation period and fastidious nature of the bacteria when cultured from clinical samples, makes definitive diagnosis difficult. This is especially true in developed countries where the disease is uncommon and usually associated with laboratory exposure or a history of travel to endemic regions such as Central America, Africa, Central Asia, the Middle-East, and the Mediterranean (3, 103, 108). Death in humans is typically associated with endocarditis, an uncommon complication (108). Human brucellosis is regarded by the World Health Organization (WHO) as a neglected zoonosis, and it is considered not only to be largely a disease of poverty in endemic countries, but also one that contributes to poverty itself through the lifelong debilitating consequences that can result from the disease (36, 111).

Brucellosis in animals:

Brucellosis is primarily a disease of animals, especially cattle, swine, camels, and small ruminants. The significance of the disease lies in both the economic losses caused by reproductive disease in the host, as well as the zoonotic nature of the disease. Generally, the most common presentation of brucellosis in animals is abortion. Animals with a high level of erythritol in their placenta such as ruminants and pigs are especially susceptible as *Brucella spp.* show a strong preference for erythritol as a carbon source and thus develop tropism for placental trophoblasts where the bacteria replicate readily often inducing abortion (112, 113). Infected placentae can contain up to 10^{13} colony forming units (CFU) per gram of cotyledonary tissue (114). Along with abortion, infected animals commonly present with retained placenta, orchitis, and epididymitis. A herd-level outbreak is often associated with an increased rate of abortions

and a decrease in reproductive efficiency (115). In the United States, the disease is notifiable in any domestic species and has been eradicated from domestic herds. Sporadic outbreaks continue to occur in the U.S. however, and of these the majority can be traced back to exposure to infected wildlife such as elk, bison, and feral swine (116, 117). These outbreaks are of significance because they threaten the U.S. pork, beef, and dairy industries if the U.S. should lose its status as a brucellosis-free country. To date, over $3.5 billion has been spent on eradication of the disease, surveillance, and efforts to prevent its reintroduction into U.S. animal agriculture (4).

Diagnosis of suspected outbreak or sporadic infection in animals is primarily based on clinical findings of mid- to late-term abortion, retained placenta, and/or metritis and serological testing via agglutination tests against the O-side chain of the *Brucella* lipopolysaccharide (LPS) or complement fixation (118). Of these, the most common screening tests are the Rose-Bengal card test and the buffered plate antigen agglutination test which use killed *B. abortus* to detect agglutinating antibodies. Raw milk samples can be screened similarly via the milk ring test in which killed stained whole bacteria are added to pooled or individual milk samples and, if present, agglutinating antibodies in the sample will form a visible ring of bound stained antigen at the fat layer in the sample. Complement fixation is used predominantly as a confirmatory test (118). It is worth noting that the conventional tests are less reliable in swine; however, the card test and buffered antigen test are the most commonly used in practice (108). Though definitive, diagnosis via culture is both uncommon and difficult as *Brucella spp.* are difficult to isolate in culture from clinical samples and are classified as biosafety-level 3 (BSL3) Select Agent organisms, thus practically and legally excluding them from being grown in most clinical laboratories (106, 119). Worldwide, *Brucella* infections are the most commonly acquired infections from laboratory settings, further underscoring the value of serological tests in

diagnosis of animal disease (118, 119). Recently, genetic tests via polymerase chain reaction (PCR) have been developed (120). These tests offer the advantage of species-specific identification; however, they rely on the organism being present in the collected sample rather than diagnosis from a serum sample, which does not require isolation of the organism itself and may be safer for veterinarians and technicians performing sampling and testing.

Wildlife reservoirs of *Brucella* display similar signs of infection to those of livestock. In the United States, infected abortion and placental products from elk, bison, and feral swine present the greatest risk of exposure to humans and other animals due to the high numbers of bacteria in these tissues and propensity of other animals to ingest such products (117, 121). Of these species, elk and bison exposures are largely limited to the Greater Yellowstone Area (GYA), whereas feral swine are present in at least 38 out of 50 states (117). While infected livestock are removed from the population via test and slaughter procedures in developed countries, wildlife species can continue to spread the bacteria through reproductive secretions. Most animals only abort their first calf or litter, though they continue to be chronically infected and can be a source to other animals and humans (113, 117). To date, no vaccine appears effective in these species against *B. suis* and complete eradication of the disease from the United States hinges on its eradication in wildlife reservoirs (1, 88, 117, 122).

Control of brucellosis through vaccination:

Control of brucellosis in domestic species has best been attained through two major principles: Surveillance by test and slaughter and calf-hood vaccination (123). Incidence of human infections has been reduced in part by pasteurization of milk and improved sanitation and worker safety in laboratory and abattoir settings. However, the best way to reduce the incidence of the disease in humans is by controlling the level of brucellosis in domestic herds, further

underscoring the significance of vaccination (96, 124, 125). Due to the role of CMI in protective immunity, all of the currently used vaccines and most promising vaccine candidates are live attenuated strains, which best induce IFNγ production, macrophage activation and cytotoxic T-cells. Currently there are three main vaccines approved for use in animals in different parts of the world, *B. abortus* Strain 19 (S19), *B. abortus* strain RB51 (RB51), and *B. melitensis* strain Rev 1 (Rev1) (88, 123). Currently there is no approved vaccine for use in humans and none of the three main animal vaccines is both safe and effective in all host species or life stages. Additionally, each of the current vaccines has at least one major drawback, including antibiotic resistance and interference with serological testing. Because of this, vaccine development continues to be a major subject in the field of brucellosis research.

The oldest brucellosis vaccine still in use today is S19 which was originally isolated in 1923. S19 is a smooth strain, which carries a spontaneously acquired deletion in the genes *eryC* and *eryD*, responsible for erythritol oxidation *in vivo* (126, 127). Erythritol is an abundant four-carbon sugar in fetal and placental tissues of ruminants and is the preferred carbon source of *Brucella spp.* (126). Though up to 91% protective against abortion in cattle, S19 has several major drawbacks. The strain is virulent in humans, poorly protective in wildlife reservoirs including elk and bison, and rarely can cause abortion in pregnant cattle (127). The biggest drawback is that the smooth LPS of S19 causes a positive result via the standard tube agglutination, card, and complement fixation tests, thus interfering with differentiation between vaccinated and naturally infected animals (128). Due to the importance of surveillance in countries with brucellosis eradication programs, S19 has largely been replaced by RB51 which does not interfere with diagnostic testing (88). S19 is still in use in some countries with large numbers of cattle including Argentina and India (127). There is little to no data available on the

efficacy of S19 in pigs and the lack of protection in elk and bison, combined with incompatibility with surveillance efforts, rules out the strain for use in wildlife reservoirs such as feral swine.

The only widely used vaccine against *B. melitensis* infection in small ruminants is the Rev1 strain which was first described in 1955 after successive passage of wild-type (WT) *B. melitensis* under selection for streptomycin resistance. The mutation causing streptomycin resistance is found in the ribosomal protein S12 gene *rpsL* (129). Demonstration of protection against WT challenge in goats in the United States was described in 1957 (130). Since that time, Rev1 has been used worldwide in sheep and goats. Tests in cattle have shown greater protection than strain S19; however Rev1 is also less attenuated and has been isolated from bovine abortion products after exposure to ewes vaccinated with the strain (131, 132). In addition to cross-protection in cattle, Rev1 also confers cross-protection to rams against *B. ovis* infection adding to the strain's value in small ruminants. However, Rev1 is more virulent than S19 to both cattle and humans and shed from vaccinated ewes causing a public health concern. Furthermore, the strain has smooth LPS making it undesirable for eradication campaigns as O-side chain antibodies confound serodiagnosis of infected animals (127).

Due to the absence of *B. melitensis* in domestic small ruminants in the United States, Rev1 is not in use. In cattle, strain S19 was the official vaccine until it was replaced in 1996 by strain RB51 (127). Strain RB51 was developed by Schurig et al. (1991) at Virginia Tech by passage of WT *Brucella abortus* 2308 on a rifampin-containing medium until a rough mutant was generated (133). A rough mutant was desired due to the inability to cause seroconversion and known efficacy of the attenuated rough *B. abortus* strain 45/20. Strain 45/20 itself is not fully rough or stable and is unable to confer satisfactory protection in cattle. RB51 was shown to be a stable rough mutant that did not cause seroconversion in vaccinated animals and which

caused a similar immune response in cattle to strain S19 (128, 133). Furthermore, RB51 is safer for use in pregnant cattle than S19, in addition to conferring similar or better protection against *B. abortus* challenge (134, 135).

Though uncharacterized at the time of implementation of the vaccine, the rough mutation in RB51 has since been shown to be caused by interruption of *wboA*, which codes the glycosyl transferase responsible for polymerization of the LPS O-side chain. The interruption is an insertion sequence termed IS711 that is present in multiple copies within the *Brucella* genome and which is generally regarded as stable (136). IS711 causes a stable frame-shift insertion within *wboA* in RB51. This frame-shift likely also affects the second known gene in the operon, *wboB* (G. Smith et al., unpublished data). Gene *wboB* is thought to assist in the same function as *wboA* however little work has been done to characterize the gene more fully (123). The *wboAB* operon is outside of the region in which the majority of LPS synthesis genes are found and it is unknown if RB51 carries additional mutations in any of these or other regions (123). However, other mutants of *wboA* in *Brucella*, while they are attenuated, are more virulent than RB51 so *wboA* is unlikely to be the sole mutation in the strain, the complete genome sequence of strain RB51 has only recently been published and the exact mutations which differentiate it from the parent strain *B. abortus* 2308 have yet to be fully explored (23, 137).

Strain RB51 is becoming accepted worldwide as the approved vaccine against brucellosis in cattle, conferring up to 100% protection against abortion (135), however it has several important disadvantages. The major disadvantage is rifampicin resistance as rifampin is one of the main antibiotics used for treatment in human infections. Also, in cattle the vaccine may cause abortion in pregnant animals and is possibly shed in milk and vaginal secretions for a short period of time before clearance (138). Strain RB51 also fails to cross protect cattle against

challenge with virulent *B. suis* making cattle susceptible to infection via exposure to *B. suis* infected wildlife (139). In cattle, these disadvantages are outweighed by the benefits of protective efficacy and compatibility with surveillance programs.

Two disadvantages of strain RB51 that are of particular concern to this dissertation are that the strain is resistant to the antimicrobial rifampin and protective efficacy of the vaccine has not been demonstrated as strongly in other species of concern, including small ruminants, elk, bison, and swine (117, 127, 140, 141). There is no approved vaccine for swine brucellosis in the U.S. or worldwide and control measures are therefore currently limited to testing and slaughtering of potentially infected animals. Strain RB51 has been shown to be ineffective in prevention of infection and abortion in domestic pigs and other vaccine candidates have not been adopted in the U.S. because of safety, efficacy, and diagnostic concerns (88, 140). Due to the unavailability of a brucellosis vaccine to pork producers and wildlife managers, and to the endemic nature of the disease in feral swine, additional disease control methods with good protective efficacy in pigs are needed (117).

Brucellosis vaccines in swine

During early testing of strain RB51, a field trial suggested that the vaccine may be protective against *B. suis* infection and abortions in swine (142); however, RB51 has not been shown to confer protection under experimental conditions and it is not considered effective in pigs (140). Strain RB51 was also isolated from a field sample of a feral pig in South Carolina that was part of an enzootically infected population used in an RB51 vaccination field trial (7). It is unknown how the individual animal was infected, since RB51 is not thought to persist beyond 4 weeks in swine. Attempts at vaccination with the older vaccine strain *B. abortus* S19 also

failed to demonstrate a significant immune response (140). In general, studies using strains RB51, S19, and other vaccine candidates using attenuated *B. abortus* or *B. melitensis* fail to demonstrate strong cross-protection against *B. suis* infection (140, 143).

Another older vaccine which has been used to some extent is *B. suis* strain 2 (S2), a smooth laboratory-adapted strain originating from China in 1953 (144). Though the S2 strain is more attenuated and potentially safer than S19, it does not produce as lasting of an immunity in the mouse model and data is lacking on the strain's efficacy in pigs (144). This, combined with its smooth phenotype, makes it unsuitable for use in countries with brucellosis surveillance programs and its current use is limited to oral vaccination in ruminants and pigs in China and the vaccine is not approved elsewhere (127, 144, 145).

Most recently, a rough strain (*B. suis* 353-1) isolated from a feral pig sample by researchers at the USDA National Animal Disease Center (NADC) showed promise as being stable and capable of inducing an immune response. However, the strain is still in the process of being evaluated for efficacy against virulent *B. suis* challenge (146). A potential drawback of strain 353-1 is that the vaccine strain was isolated from mucosal swabs in one or two pigs that were vaccinated conjunctivally, but intramuscularly vaccinated animals did not shed the vaccine. *B.suis* strain 353-1 induced both a humoral immune response and CMI, however previous research has shown that correlation between the immune response induced by the vaccine and protection against challenge is inconsistent in wildlife species, so further testing is needed (146). An effective vaccine against brucellosis in swine is highly desirable due to the lack of a widely accepted vaccine for domestic pigs as well as the capability of feral pigs to spread the disease to both humans and livestock. Feral pigs are the sole remaining wildlife reservoir of *Brucella* in the continental United States outside of the Greater Yellowstone Area and an effective vaccine,

combined with feral swine population control would be a valuable tool in eradicating the disease from the US.

Feral Pigs as a Nuisance Species and Reservoir of Disease

Feral swine in the United States, past and present

Feral swine (*Sus scrofa*) are the largest invasive animal species in the United States and the second most costly vertebrate pest behind only rats and mice in terms of annual damages to agriculture, property, and the environment (147). Also referred to as feral pigs, and wild boar, feral swine in the U.S. are descended from the European wild boar (*Sus scrofa*) and naturalized domestic pigs and as a result are not a homogenous population. European wild boars and domestic hogs have been intermittently released both accidentally and intentionally as a food and game species in North America since as early as 1539 and have been naturalized into the modern feral pig (148). Since their initial introduction and additional naturalization of domestic strains, feral swine have become an invasive species of major concern and their numbers in the United States have grown to an estimated 4 million animals (149, 150). Feral swine are prolific breeders, having litters of 5-8 piglets and being capable of 2 litters in a calendar year, thus contributing to their abundance and destructive impact (151, 152). Free-ranging pig populations are now present in at least 39 states and cause extensive damage by rooting up crops and breaking through livestock fences to consume animal feed and prey on small livestock such as young sheep and goats (152, 153). Populations are highest in the major agricultural states of California, Florida, and Texas where they have been labeled a nuisance species and extensive efforts to control their population such as state-sponsored hunting and trapping have taken place. However, these efforts

have been constrained by budget and efficacy concerns and other population control methods are needed (154-157). Particularly troubling to state agencies is the recent expansion of wild pigs into northern states like Michigan, where feral swine were not present thirty years ago. Feral swine now inhabit 72 of 83 counties in the state. The Michigan Department of Natural Resources has since declared them an invasive species and adopted drastic legislative measures outlawing their possession in the state as of April 01, 2012 (158). Virginia is another state which has seen the recent expansion of its feral swine population (159). The population in Virginia in 2009 was limited to a few isolated herds likely originating in North Carolina and Tennessee, but since that time has grown to include over 30 counties (160). The Virginia Feral Hog Stakeholders Group has since formed with the backing of the USDA and Virginia Department of Game and Inland Fisheries (161). Furthermore, population growth of wild hogs in the U.S. is not confined to northward expansion into new states; nationwide it is estimated that the population of feral swine has quadrupled in the past 10 years making population control a priority to wildlife pest management programs (162).

Stakeholders in government, industry, and academia have demonstrated concern over the impact of feral swine in the United States and addressed the need for additional control tactics (152, 163-172). The predominant control method, hunting, appears to be ineffective in reducing the population of feral swine and in some states where recreational hunting has been encouraged, populations have expanded as a result of intentional releases on hunting grounds and damages have not been reduced (156, 163). Nationwide, the annual damages caused by feral swine are conservatively estimated to be around $1.5 billion, and these damages have increased over time (147, 149, 173). In Texas alone, annual property and agricultural damages amount to approximately $400 million and the state has enacted radical depopulation measures citing

estimates that for every dollar spent on control of feral hogs, $7.50 is saved in agricultural product (174). Like other states' agencies, the Texas Parks and Wildlife Commission has stated that the feral swine problem continues to worsen and that additional resources and control methods are needed. The North Carolina State Legislature passed a bill in 2009 redefining hunting regulations for swine and appropriating funds for the study of feral swine control (157, 166). At the federal level, the Food, Conservation, and Energy Act of 2008 (the 2008 Farm Bill) recognizes feral swine as a threat to the domestic swine industry and to other livestock and mandates that the USDA continue disease surveillance and control measures as they pertain to free-roaming pigs (175). In a demonstration of industry support for feral swine control measures, the National Pork Producers Council and the National Pork Board voiced their support for that section of the bill and confirmed feral swine as an industry concern for disease transmission to domestic herds (167, 176). In recent years, wild pig populations have been documented to be the source of a variety of infections to both humans and livestock. This fact, combined with their potential for both ecological and economic damage, makes improving control methods for feral swine a priority in both wildlife control and infectious disease research.

Disease concerns in feral swine

Feral swine are proven carriers of several important diseases of domestic pigs, including influenza, leptospirosis, trichinosis, classical swine fever, hepatitis E, pseudorabies and brucellosis (24). Due to its established prevalence in feral populations and high transmissibility, one of the diseases of greatest concern to producers is brucellosis (177). Cases of transmission of *B. suis* to humans from feral pigs have been documented both in the United States and abroad (178). The practice of hunting wild boar is especially common in the Southern United States, putting hunters at increased risk for contracting brucellosis from swine (24). Two of the states in

which cases of brucellosis related to hunting activities have been confirmed are Florida and South Carolina (178). Feral swine have also been implicated as the source of infection in brucellosis positive cattle and may harbor and transmit both *B. suis* and *B. abortus* (7, 179).

In addition to carrying brucellosis, feral swine also maintain several other significant zoonotic diseases which have had a demonstrated impact on public health in the United States. For example, a recent nationwide *E. coli* O157:H7 outbreak which sickened 205 people and resulted in three deaths in 2006 has been attributed to fecal contamination of spinach in California by a feral swine herd (180, 181). Another zoonotic disease concern involving feral swine of recent importance is the ability of swine to harbor influenza A viruses. Pigs are known to be infected with influenza belonging to porcine, avian, and human strains, thus creating an opportunity for genetic reassortment similar to that which created the pandemic 2009 H1N1 strain. Swine are also thought to have been the intermediate host in the 1918 influenza pandemic responsible for 50 million human deaths worldwide (182, 183). In the United States, a 1974 influenza outbreak in New Jersey was directly attributed to pigs (184). Currently, both feral and domestic swine populations in the United States harbor multiple influenza subtypes including the H1N1 2009 pandemic strain (which was not detected prior to the human outbreak), swine H1N1, H3N2, and newly reassortant H1N2. The H1N1 subtype is historically the dominant domestic swine influenza; however, recently the reassortant H3N2 subtype has become dominant which harbors human, avian, and swine-strain components (183, 185, 186). Hall et al. (183) recently conducted a serological survey of feral swine in different regions of the United States and identified H1N1 and H3N2 seroconversion in as many as 14% of animals. More recently, Corn et al. (185) detected antibodies to swine influenza virus in 90.7% (n=119) of feral animals in North Carolina including H3N2, human-like H1N1 and reassortant H1N2-like H1N1. This

survey was deliberately conducted in areas where both intensive and transitional, or “backyard” hog farming is common and suggested that the feral population became infected by domestic animals. Once established in the feral population these influenza strains not only persist but also pose an increased risk for reassortment with avian influenza due to the increased exposure of wild animals to infected birds and risk of simultaneous infection with multiple strains (183, 185).

Feral swine also carry major diseases with significant potential economic impact to agriculture, including tuberculosis and pseudorabies. Pseudorabies (also referred to as Aujeszky’s disease) is caused by the alphaherpesvirus pseudorabies virus (PRV). Pseudorabies was eradicated from the domestic swine herd in 2004 after a 15 year campaign through the USDA-APHIS PRV Eradication Program (187). In pigs it is mainly a respiratory and reproductive disease causing abortions and stillbirths in endemic herds. In naïve herds the disease causes worsened reproductive and respiratory disease in all age groups and serious central nervous system (CNS) signs as well. Since the national domestic herd is naïve post-eradication, an outbreak could be responsible for high morbidity and mortality via reproductive, respiratory, and CNS disease (187). Cattle, small ruminants, cats, and canines may also be infected causing fatal CNS disease with 100% mortality. The seroprevalence of antibodies against PRV in feral swine is as high as 38% making exposure to wild pigs a serious threat to US Agriculture (188). Another disease, which has largely been eradicated from the United States is bovine tuberculosis (TB). Though some positive herds periodically appear in the upper Midwestern U.S. due to exposure to white-tailed deer, TB has been eradicated after an extensive test and slaughter campaign. Feral swine have not been shown to cause outbreaks in cattle in the U.S. and the prevalence of TB in American feral populations is poorly studied; however, in Europe up to 50% of wild boar are positive for TB and the incidence appears to have increased in

the past fifteen years (24, 189). Furthermore, European wild boar are thought to be a source of infection in cattle in previously TB-free herds (189). These and other diseases of feral swine have the potential to cause serious damage to U.S. agriculture. This problem is further exacerbated by the increase in the feral swine population and trend towards "backyard" and outdoor operations for raising domestic pigs. In light of the failure of current control efforts in feral pigs, additional tools are needed to both mitigate the potential for disease transmission to humans and livestock and to reduce the feral pig population.

Immunocontraception and wildlife control

Due to the prolific nature of feral pigs and demonstrated lack of efficacy with hunting and trapping efforts alone, additional management tools are needed to control the population of feral pigs. One potential integrated pest management tool, which has been explored to a limited extent, is the use of infertility agents with the aim of reducing population growth via chemical or immunological methods (190, 191). Infertility agents are desirable both as an added tool to manage wildlife populations in general and in situations where lethal means of controls are either ineffective, impractical, or unavailable due to public safety and animal welfare concerns. Examples of effective population control in situations where lethal methods would not be acceptable are via the use of immunocontraceptive agents to control white-tailed deer (*Odocoileus virginianus*), wild horses (*Equus caballus*), grey squirrels (*Sciuris carolinensis*) in Clemson, South Carolina, and fox squirrels (*Sciuris niger*) in Davis, California (192-195). While studies exploring the use of toxic chemical means of fertility control in feral pigs (such as with candidate the ovotoxin ERL-4221) are limited and have been unsuccessful (190), multiple

immunocontraceptive biological compounds have been shown to have an effect in feral pigs (196).

Principles of Immunocontraception

There are four basic approaches to contraception: surgical, mechanical, pharmacological/chemical, and immunological. Of these, the newest and most applicable to wildlife control is immunological contraception (197). Immunocontraception involves the use of an antigenic peptide conjugated to a reproductive hormone or other compound to elicit an immune response against the endogenous compound. Of these compounds, the most promising are porcine zona pellucida (PZP, mainly used in cervids) and antigen-conjugated formulations of gonadotropin-releasing hormone (GnRH) (198). GnRH-based vaccines are readily generated via recombinant DNA technology and in one study, a GnRH-based vaccine outperformed a PZP-based vaccine in a rodent model (199). GnRH (also known as luteinizing hormone-releasing hormone, LHRH) is a decapeptide hormone secreted by the hypothalamus to act on GnRH receptors on endocrine cells in the anterior pituitary gland. In response to GnRH stimulation, pituitary cells secrete the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which act on the gonads to control reproductive function. As a result, GnRH is the highest level of hormonal control over reproduction, making it a rational target for disruption of fertility (191, 197, 200). When an immune response is mounted against endogenous GnRH, estrogen and testosterone levels decrease, reproductive activity decreases, and gonadal development may regress (201).

Early studies of fertility control via disruption of endogenous GnRH involved administration of GnRH-like peptides with specific amino acid substitutions for competitive antagonism at the receptor level. While shown to be effective in suppressing reproductive

function in beagles, this technique involves daily administration and is thus impractical for use in a wildlife control scenario (202). Subsequently, immune-based methods were tested using tandem repeats of GnRH, GnRH with single amino acid substitutions or GnRH conjugated to various antigenic carrier peptides and delivered as adjuvanted subunit preparations (199, 203-205). In the United States, GonaCon, GnRH conjugated to the antigenic peptide keyhole limpet hemocyanin (KLH) and delivered as a purified subunit in an oil-based adjuvant containing killed *Mycobacterium avium*, is conditionally approved for the control of white-tailed deer. GonaCon was developed at the USDA-APHIS National Wildlife Research Center (NWRC) and is the only approved immunocontraceptive for wildlife control in the U.S. It has been tested in many species, including feral swine, though its field use is currently restricted to control of white-tailed deer only (201, 206, 207).

Immunocontraception in feral swine

Several immunocontraceptives, including the GnRH-KLH preparation GonaCon, have been tested in both domestic and feral swine (196, 201). Domestic pigs have been used as a model for feral swine and immunocontraceptive vaccines also have the potential to be used in commercial production as an alternative to surgical castration to control boar taint and promote growth (208, 209). In feral pigs, immunocontraceptives may be useful to help control the growth of the population. They may also aid in reducing disease transmission as two of the major diseases of concern, brucellosis and pseudorabies, are spread via sexual activity and oronasal contact with reproductive discharges (201).

GonaCon, a GnRH peptide monomer conjugated to KLH and delivered parenterally in a strong adjuvant, has been tested in feral and domestic pigs under different conditions. Using domestic pigs as a model, Miller et al. (2003) found that a single dose followed by a booster

injection prevented estrus in 100% of gilts and a single high dose alone prevented estrus in 70-90%. In boars, both single and double administration led to reduced serum testosterone and testicular size versus controls receiving adjuvant alone (208). In captive feral pigs, Killian et al. (2006) showed that a single administration of a high dose of GonaCon (using 1 or 2 grams of peptide) was effective in preventing estrus in females. In this study, males were more responsive to the relatively lower dose (1g), but less responsive to the vaccine over all (201). In a separate study in the United Kingdom, Massei et al (2008) found that antibody titers in multiparous sows against endogenous GnRH peaked between 2 and 6 weeks post-administration of a single dose and that titers remained high out to 12 weeks. It took approximately 4 weeks for fecal progesterone levels to reach basal levels (206). Massei et al. (2012) also evaluated the longer-term effects of GonaCon and observed captive females over 4-6 years for reproductive and behavioral effects of the vaccine (210). Behavioral effects were considered in the study to determine if immunocontraception disrupted social hierarchy and other aspects of pig behavior. They were assessed via 3-hour observation sessions every other week for the duration of the study in which various behaviors were recorded and agonistic interactions between individuals were charted. It was found that while anti-GnRH titers decreased over time, all animals had a significant titer at 6 years post-administration of a single dose of GonaCon and in that time only one litter was produced in total by the twelve females studied. No changes in body weight or behavioral effects were observed (210).

GnRH itself is a B-cell peptide, which is non-immunogenic without conjugation to some other antigen, especially a T-cell antigen. In GonaCon and other GnRH-fusion based vaccines, the conjugated peptide is responsible for generating the immune response against both heterologous antigen delivered in the vaccine and subsequently against endogenous GnRH (196).

Another recombinant GnRH-based vaccine which takes advantage of this principle and that has been tested in pigs is the “Talwar recombinant” (196, 211, 212). The Talwar Recombinant is a multimeric fusion peptide of GnRH monomers (mGnRH) interspersed with B and T cell epitopes to produce both short-term IgM and long-term IgG antibodies to the peptide. The specific epitopes were chosen for their “promiscuous” nature as universal immunogens in order to cause a response in multiple species. The epitopes are 13-21 amino acids long and include the dominant antigenic peptides of *Plasmodium falciparum*, tetanus toxoid, respiratory syncytial virus, and measles virus, each separated by a GnRH decapeptide and 3 amino acid linker sequence (G-S-G) for a total of 5 GnRH monomers within the multimeric mGnRH. Like GonaCon, mGnRH has been delivered as a subunit vaccine however, mGnRH is recombinant and can therefore be produced at lower cost (196).

Originally developed for use as a treatment for prostate disease in human medicine, the Talwar recombinant (mGnRH) has been shown to cause prostatic atrophy in rats and has been tested in pigs as a lower cost alternative to GonaCon (196, 211). Miller et al. (2006) found that a single administration of mGnRH delivered as a subunit vaccine in a strong adjuvant was sufficient to cause a significant anti-GnRH titer. Three out of five gilts given a single administration either failed to conceive or would not stand to be bred at any time during the study and zero of five gilts receiving a booster vaccination at 4 weeks stood to be bred versus all control individuals (196). The advantages of mGnRH are that the recombinant epitope-only antigen preparations are lower cost than other subunit immunocontraceptives and that the small sized molecule (approximately 17kDa) is readily expressed and purified from *E. coli* in large quantities (196, 212, 213). Though the results using mGnRH in a pilot study in pigs and other

studies using GonaCon were promising, further testing remains to be done to assess the viability of the subunit preparations as an option for feral pig population control in field conditions.

Though promising, subunit vaccines have several major disadvantages when used for wildlife control, the most significant being cost (196). Other disadvantages include that they are limited to parenteral delivery which is impractical in a field situation, and that they require a very large dose and potent adjuvant, which could cause injection site reactions. In the past, *Brucella* vaccine strains have been engineered to deliver homologous and heterologous antigens capable of eliciting protective titers against a number of diseases (63, 74, 214). The small size of mGnRH and stable maintenance on plasmid DNA make it amenable to delivery via a modified live *Brucella* platform. The major advantages of delivery via modified live vaccine are that they generally do not require an adjuvant to be effective, allow flexibility for route of vaccination, and are extremely low cost relative to subunit preparations (88, 94). The work of this dissertation details development and testing of a novel brucellosis vaccine and evaluating its suitability as a platform for delivery of modified mGnRH as a candidate immunocontraceptive vaccine.

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CHAPTER 2: DEVELOPMENT OF A MARKERLESS RECOMBINANT *BRUCELLA SUIS* VACCINE CAPABLE OF EXPRESSING HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

Abstract

Feral swine are a major nuisance species in the United States and cost around $1.5 billion each year in agricultural, environmental, and personal property damages. In the last ten years the population of feral swine has grown exponentially and additional population control methods are needed. Feral swine are also the most widespread carriers of the zoonotic disease brucellosis, which threatens both livestock biosecurity and public health. Currently, there is no approved vaccine against brucellosis in pigs. This work details development of a live bacterial antigen delivery system directed at swine using *Brucella suis* VTRS2 as a novel platform. Strain VTRS2 can be employed to deliver the multimeric gonadotropin-releasing hormone (mGnRH) immunocontraceptive antigen without the use of antibiotic resistant markers. The strain was created by deletion of the LPS biosynthesis gene *wbo*A as well as the *leu*B gene required for leucine biosynthesis inside the nutrient-depleted intracellular environment, i.e. the macrophage occupied by *Brucella* within the *Brucella* Containing Vacuole (BCV). The vaccine strain *B. abortus* RB51, which is attenuated in part by *wbo*A mutation, is widely used in cattle, however strain RB51 is rifampin resistant and has not been shown to have protective efficacy in swine. It was hypothesized that strain VTRS2 will survive within mice for sufficient time to induce a protective immune response and function as a candidate vaccine against *B. suis* challenge. Furthermore, it was hypothesized that the mGnRH antigen can be delivered using the pNS4 plasmid, which expresses *leuB* under its native promoter, thus maintaining the plasmid in strain

VTRS2 under leucine-deficient conditions. Initially these recombinants were tested in culture to confirm the rough phenotype and determine the mGnRH expression capability of the VTRS2 strains as a prerequisite to testing the candidate strains in the mouse model. An improved vaccine against brucellosis in swine, as well as one which confers immunocontraception without the use of antibiotic resistance, could become an important tool in the management of feral swine.

Introduction

Brucellosis, caused by members of the Gram negative bacterial genus *Brucella*, is among the most prevalent zoonotic diseases worldwide (1). In the United States, the disease has been eradicated from domestic swine and cattle herds; however, the disease still exists in wildlife reservoirs including elk (*Cervus elaphus*), bison (*Bison bison*), and feral swine (*Sus scrofa*). Among these, feral swine are the most widespread in the U.S. and they have been attributed to recent outbreaks of the disease in domestic herds as well as zoonotic infections in humans (2, 3). The population of feral swine, which are an invasive species in the U.S., has roughly quadrupled in the last decade and accounts for roughly $1.5 billion in damages annually nationwide (4, 5). They have been shown to carry *Brucella suis*, which has a host preference for swine but can also infect cattle, as well as *B. abortus* which has a host preference for cattle. All *Brucella spp.* carried by feral swine are also capable of causing infection in humans (2). Feral swine not only cause widespread economic loss, but they are also increasingly in contact with both humans and domestic livestock. This increased contact raises their potential to spread brucellosis, as well as other zoonotic diseases such as leptospirosis and influenza (6).

Currently, there is no approved vaccine against swine brucellosis. The vaccine approved for cattle in the United States, *B. abortus* strain RB51, is poorly protective against *B. suis* in the

mouse model and not considered efficacious in swine (7-9). However, RB51 has several important advantages over other candidate vaccines. Most importantly, the strain has a stable rough phenotype as a result of "IS711" insertional inactivation of the gene *wbo*A. The *wbo*A gene codes for the enzyme glycosyl transferase, which is responsible for polymerizing the O-side chain of the *Brucella* LPS. In addition to attenuating the strain, the rough phenotype also results in vaccinated animals testing negative for *Brucella* exposure on serological assays due to the lack of the dominant LPS O-side chain antigen (9, 10). This is a critical feature of strain RB51, as test and slaughter were essential in eradicating the disease from domestic species in the United States. In cattle, being able to differentiate infected individuals from vaccinated ones (DIVA) is of high importance for disease surveillance (11, 12). However, strain RB51 also has some disadvantages which reduce its suitability for use in feral swine. In addition to being poorly effective in pigs, RB51 is rifampin resistant which could have undesirable environmental repercussions i.e. potentiating the spread of an antibiotic resistance gene. Strain RB51 also is thought to have additional mutations which have not yet been fully described in published literature, though the genome has recently become available (11, 13). This work describes development of a rough strain of *Brucella suis*, VTRS2, which has defined deletion mutations in genes *wbo*A and *leu*B. Deletion of *wbo*A confers the rough phenotype, while deletion of *leu*B renders the strain unable to biosynthesize leucine to grow in a leucine-deficient environment and allows the strain to maintain the markerless family of plasmids, pNS4, which complement the *leu*B gene (14). Inclusion of *leu*B on pNS4 allows the plasmid to be used to overexpress heterologous and homologous antigens in VTRS2 without the use of an antibiotic resistance marker. One such antigen is the candidate immunocontraceptive mGnRH.

Immunocontraceptive vaccines have been proposed as an additional tool for wildlife management agencies to control nuisance species (15, 16). Current vaccines stimulate an immune response against endogenous reproductive hormones such as gonadotropin releasing hormone (GnRH) by conjugating the hormone with an antigenic carrier (15); however, most of these vaccines are delivered as subunit preparations which are not cost effective to produce and would be highly expensive to use in feral swine (17). It has been shown previously that *Brucella* vaccine strains can be used to deliver antigen via the pNS4 family of plasmids. Herein, an attenuated *B. suis* vaccine was engineered to express mGnRH, a small recombinant antigenic form of GnRH originally developed by the Talwar Research Institute (New Delhi, India), for expression in *E. coli* and purification for subunit delivery (Figure 2-1) (17-19).

The candidate platform strain VTRS2 was created using the cre-lox recombination system, which allows stable deletion mutations without the introduction of permanent antibiotic resistance genes (14, 20). The mGnRH antigen was codon-optimized for expression in *Brucella* (mGnRHb) and transformed into VTRS2. A recombinant fusion peptide of two known *Brucella* antigens was also expressed in VTRS2 to demonstrate the ability of the strain to express homologous antigen. The strains were then characterized to confirm that they remained rough and unable to synthesize leucine without complementation with pNS4 and that they were able to express the mGnRH as well as the fused *Brucella* antigens.

Materials and Methods

Creation of the candidate vaccine strain VTRS2

Strain VTRS2 was created via sequential cre-lox recombination deletion (20) of genes *wbo*A (encoding a glycosyl transferase) and *leu*B (encoding isopropyl malate dehydrogenase)

from the virulent reference strain *B. suis* 1330. First, 416 bp of the LPS O-side chain polymerization gene *wboA* were deleted from *B. suis* 1330 using the recombination plasmid pGEMwboA:LCL (Figure 2-2). The plasmid was generated in *E. coli* DH10b using pGEM-3z, an upstream (UP) PCR fragment of *wboA* (725 bp), the loxP recombination cassette including a chloramphenicol-resistance marker, and a downstream (DWN) wboA fragment (472 bp) beginning 416 bp downstream from the UP fragment to generate the deletion (see Table 2-1 for primers). The pGEM-3z plasmid (Promega) was used to deliver the mutation cassette as it is not maintained in *Brucella spp.* and therefore chloramphenicol-resistant clones must contain the recombinant insertion into the genome.

After confirmation of the plasmid sequence (Biocomplexity Institute of Virginia Tech, BCI; Blacksburg, Virginia), pGEMwboA:LCL was transformed into competent *B. suis* 1330 cells via electroporation. Competent cells were made via harvesting the bacteria from agar plates into phosphate buffered saline (PBS, 5 ml per plate) and centrifugation at 2°C in a tabletop centrifuge at approximately 2,000xg. The pellets were then washed twice in ice-cold sterile water and frozen in 65 µl aliquots prior to transformation. After electroporation, the competent cells were incubated in 600 µl super optimal broth with catabolite repression (SOC) media overnight at 37°C. Transformants were plated onto tryptic soy agar (TSA) containing chloramphenicol (Cm, 17.5 µg/ml) and incubated for 48-72 hours and 10 colonies were selected and subcultured further onto TSA+Cm. The colonies were also plated onto ampicillin (Amp, 50 µg/ml) to ensure the clones had been cured of the amp^R containing pGEM-based plasmid. Clones were then screened for roughness by crystal violet staining and clumping in the presence of acriflavine. A rough, Cm-resistant Amp-sensitive isolate (*B. suis wboA*:loxPcm) was then confirmed by PCR using the wboAIf and wboAIIr primers (Table 2-1). Any material removed from the BSL-3

laboratory for sequencing was confirmed to be free of viable bacteria via established protocols of culturing in TSB or on TSA for a minimum of 6 days at 37C. After mutation confirmation by sequencing, competent cells were made and transformed with the *cre*-containing plasmid pCM158 (20). Transformants were grown on TSA containing kanamycin (Kan, 50 μg/ml) and ten colonies were selected for screening for Kan resistance (Kan^R) and Cm susceptibility (Cm^S), thus indicating recombination had taken place to remove the Cm cassette from the bacterial chromosome. Any Kan^RCm^S colonies were checked for colony morphology and roughness was confirmed in seven colonies that had been cured of the Kan^R plasmid by successive passages on antibiotic-free TSA. Screening for Kan^S after three passages resulted in strain *B. suis* Δ*wboA*. After creation of the rough mutant of *B. suis,* the strain was made into a leucine auxotroph by mutating the gene *leuB*. This mutation allows *leuB*-containing plasmids to be maintained which contain genes that code for immunocontraception or protective antigens of either *Brucella* or other pathogens without potentiating the spread of antibiotic resistance into the environment (9, 14).

The leucine biosynthesis gene *leuB* was mutated by deletion of 574 bp from *B. suis* Δ*wboA* in the same manner as described above using the plasmid pGEMleuB:LCL (Figure 2-3) in the protocol described in Rajasekaran *et al* (14) but using updated primers for *B. suis* and a Cm-resistance based loxP cassette versus a gentamicin based cassette in keeping in accordance with CDC regulations for antibiotic use in select agents. The use of chloramphenicol and kanamycin antibiotic resistance genes in intermediate strains was approved by CDC and all intermediates were destroyed after confirmation of markerless mutation in the resulting deletion mutant strain upon transformation with pCM158. Likewise, mutants transformed with the kanamycin resistance-containing plasmid pCM158 were cured of the plasmid via successive

passage on TSA lacking antibiotic and all strains were confirmed to contain no exogenously introduced antibiotic resistance markers. Once leucine auxotrophy had been confirmed by lack of growth on *Brucella* minimal medium without leucine (BMML) the new strain *B. suis* Δ*wboA* Δ*leuB* (VTRS2) was phenotypically and genetically characterized prior to generating competent cells to accept *leuB* complemented pNS4 plasmids.

Phenotypic and Genotypic Characterization of VTRS2

Confirmation of the rough phenotype of VTRS2 was accomplished via three methods: the acriflavine dye test, crystal violet test, and the serum agglutination test (21). To perform the acriflavine test, neutral acriflavine was freshly diluted 1:1000 in distilled water, 30 µl was placed on a glass slide, and 2-3 colonies of the candidate strain were mixed in the solution with a sterile loop. Rough organisms have a charged outer membrane and therefore clump in neutral acriflavine and cause the solution to clear whereas smooth organisms stay in suspension. The *wbo*A mutant *B. suis* strains were tested and compared with *B. suis* 1330 and strain RB51 as smooth and rough controls, respectively.

For the crystal violet test, crystal violet was prepared to a dilution of 1:2000 in distilled water. The crystal violet solution was flooded over a TSA plate containing either the candidate mutant strain or a control strain and assessed for uptake of the dye by rough colonies (22). The third test, serum agglutination, was performed using mouse monoclonal antibody (Bru38) serum against the O-side chain of *Brucella* (21). Smooth colonies strongly agglutinate when mixed with the serum versus rough strains lacking the O-side chain.

Confirmation of the leucine deficient phenotype was achieved via side-by-side cultures of strain VTRS2 and the reference strain *B. suis* 1330 on TSA and *Brucella* Minimal Medium (BMM) plates deficient in leucine (BMML). BMM is a defined medium which was prepared as

described previously (23), but without the addition of leucine to generate BMML, which was used to confirm the inability of the *leu*B deletion mutant strains to biosynthesize leucine.

PCR and sequencing of the mutant strains were performed to genotypically confirm the deletion mutations. Sequencing was performed by DNA extraction using QIAamp DNA mini kit (Qiagen) and submission to the Biocomplexity Institute of Virginia Tech (BCI)) for Sanger sequencing. Primers used for sequencing included the UP and DWN cre-lox mutagenesis primers as well as additional primers used to ensure the entire region was reliably sequenced (Table 2-3). The AMOS PCR system for identification of *Brucella* species was also used as an additional tool to ensure all strains used were derived from *B. suis* (24).

Growth curve in culture was performed to compare if there was a growth defect attributable to the mutation. Briefly, 25-ml cultures of wild-type *B. suis* 1330, VTRS2, or VTRS2 containing the pNS4 expression plasmid for mGnRH or RicA/SOD were prepared in tryptic soy broth (TSB). Turbidity was assessed by measuring Klett Units on a Klett-Summerson photocolorimeter at 0, 8, 24, 32, 48, 72, and 120 hours post-inoculation in duplicate.

Creation of pNS4/mGnRHb and expression in VTRS2-mGnRHb

To create the immunocontraceptive vaccine strain VTRS2-mGnRHb, strain VTRS2 was transformed with the mGnRHb containing plasmid pNS4/*trc*D-*mGnRHb* (Figure 2-4). Plasmid pNS4*trc*D contains the gene *leu*B with its native promoter, which allows selection for maintenance of the plasmid in the Δ*leu*B strain VTRS2. The plasmid also contains the *trc*D hybrid promotor, which has been shown to constitutively express recombinant antigen at high levels (25, 26). The antigen chosen for immunocontraception was mGnRHb. The original mGnRH construct developed for use in *E. coli* could not efficiently be expressed by *Brucella*, so codon optimization was performed using OptimumGene Codon Optimization Analysis

(GenScript, Inc) to optimize the mGnRH sequence for *B. suis* 1330 codon usage without changing the resulting translated sequence of the mGnRH peptide. The resulting sequence, *mGnRHb* (Figure 2-5), was synthetically generated (GenScript, Inc) and cloned into pNS4 behind a 6xHis tag using BamHI and Acc651 restriction enzyme digestion of the multiple cloning site (MCS) and ligation. Following insertion of *mGnRHb* into pNS4*trc*D to create pNS4/*trc*D-*mGnRHb*, the insertion sequence was confirmed via PCR and sequencing (for primers see Table 2-4). All cloning was performed using the leucine auxotrophic *E. coli* strain HB101 and selection was made on BMML as described below.

After confirmation of insertion of the immunocontraceptive sequence into pNS4, the resulting plasmid pNS4/*trc*D-*mGnRHb* was transformed into strain VTRS2 and incubated overnight in SOC media. After overnight incubation the transformants were pelleted at approximately 2,000xg for 30 minutes and washed 2x with BMML broth to remove any residual leucine from the SOC media. Transformants were then plated on BMML agar plates and incubated at 37°C for 72 hours. Five colonies were then selected at random and subcultured onto BMML agar and into 10 ml BMML broth for plasmid extraction. After 48 hours at 37°C, the broth cultures were pelleted at 2,000xG for 30 minutes and plasmids extracted using the QIAprep Spin Miniprep kit (Qiagen). All plasmids were tested for the presence of the mGnRHb insertion via PCR and one isolate that was PCR positive was chosen for sequencing (see Table 2-4 for primers).

After sequence-confirmation of the strain VTRS2-mGnRHb, the candidate multivalent immunocontraceptive brucellosis vaccine was tested to determine expression of the immunocontraceptive antigen via Western blot. Western blotting was performed by pelleting 7.5 ml of 48h BMML broth cultures of VTRS2-mGnRHb and VTRS2 containing pNS4 empty

expression vector plasmid as the negative control. Pellets were resuspended in 40ul of 10mM Tris-HCl with 5% β-mercaptoethanol and lysed in a boiling water bath for 15 minutes and then mixed 1:1 with Laemmli SDS-PAGE sample buffer for SDS-PAGE electrophoresis. The separated proteins were then transferred onto a nitrocellulose membrane using a semi-dry transblotter transfer apparatus (BioRad). The membrane was then washed three times in PBS + 0.05% Tween 20 (PBST) and blocked for 1 hour in PBST+5% skim milk. After blocking, the membrane was washed as before then probed with Anti-His-HRP antibody (Abcam) overnight at 4°C in blocking buffer. After incubation with antibody, the membrane was washed and His-tagged antibodies detected via hydrogen peroxide-based development and exposure to standard radiographic film or colorimetric development using hydrogen peroxide and chloronaphthalene.

Creation of pNS4/RicA-SOD and expression in VTRS2-RicA/SOD

To demonstrate the ability of strain VTRS2 to express *Brucella* antigens in pNS4, a fusion peptide was created from the known *Brucella* virulence factor antigens RicA and SodC to form the plasmid pNS4/RicA-SOD (Figure 2-6). SodC is a copper-zinc superoxide dismutase (SOD) encoded by the gene *sodC* that has been previously shown to enhance the protection of strain RB51 in the mouse model for brucellosis vaccination (9, 27). RicA is a type-4 secretion system (T4SS) effector molecule thought to play a role in disrupting vesicular trafficking through a binding interaction with the mammalian GTPase Rab2, an important step in the virulence pathway of *Brucella* (28, 29). As an effector molecule of the T4SS, RicA is secreted by *Brucella spp*. upon entry into the macrophage and thus could serve both as an inducer of cell mediated immunity as well as a delivery mechanism for other antigens through translational fusion. The plasmid pNS4/RicA-SOD was generated by amplification of *ricA* (0.55 kb) minus the stop codon from chromosomal *B. suis* 1330 DNA and ligating it behind the native

constitutive *Brucella groE* promoter and 6xHis tag in pNS4 using BamHI and SacI restriction enzyme digestion and ligation. Then, a 0.525 kb fragment of *sodC* without its putative upstream periplasmic signal sequence was amplified along with a flexible 9 base-pair linker (G-S-G) sequence, the same sequence used between the epitopes in mGnRH. The putative signal sequence was removed to prevent cleavage of the fusion peptide during post-translational processing. The resulting GSG-SOD fragment was cloned in-frame behind *ricA* using SacI and XbaI restriction enzyme digestion and ligation to generate pNS4/RicA-SOD (for primers, see Table 2-5, Figure 2-6). After PCR and sequencing confirmation of an in-frame fusion, the plasmid was transformed into competent VTRS2 and plasmid-containing colonies were selected as described above.

For confirmation of expression and to test the hypothesis that the RicA-SOD fusion peptide would be excreted via the T4SS, two series of Western blots were performed. The first was performed as described above for mGnRHb to confirm expression. Then, to determine if the antigen was secreted into the culture medium, precipitated supernatant protein extracts of active culture were screened for the presence of the peptide. To do so, 5 ml cultures of either VTRS2/pNS4 or VTRS2/RicA-SOD were grown 12 hours (early LOG phase) in leucine-deficient *Brucella* Minimal Medium. Then, 1 ml of each culture was removed as the intracellular control and centrifuged at 12,000xg (high speed) in a microcentrifuge. The pellet was then resuspended in 40 ul of 10mM Tris-HCl with 5% β-Mercaptoethanol and lysed in a boiling water bath for 15 minutes and then mixed 1:1 with Laemmli SDS-PAGE sample buffer and placed in the -20°C freezer for storage until the culture supernatant protein extract was ready for SDS-PAGE. The remaining 4 ml of each culture were spun for 30 minutes at approximately 2,000xg in a tabletop centrifuge and then the supernatant was transferred to a new centrifuge tube and

spun a second time to ensure pelleting of all cellular debris. The supernatant was again transferred to a new tube and the protein fraction precipitated in 10% trichloroacetic acid (TCA) for 12 hours at 4°C. After precipitation, the supernatant was again centrifuged and the pellet washed once in cold acetone to remove any residual TCA. The acetone was then decanted and the remainder allowed to air dry before resuspending the pellet in 40 ul of Laemmli SDS-PAGE sample buffer. After SDS-PAGE, protein bands from the polyacrylamide gel were transferred onto a nitrocellulose membrane, blocked in skim milk, and incubated with mouse anti-His-HRP conjugated antibody (Abcam) overnight in skim milk at 4°C as described for mGnRHb. The probed film was then exposed and developed on conventional X-ray film to screen for the presence of a ~38 kDa band in the VTRS2/RicA-SOD extracted culture supernatant sample to indicate secretion of the chimeric RicA-SOD effector protein. In addition to using strain VTRS2 with only pNS4 plasmid as a negative control, the absence of an unknown ~36 kDa protein of *Brucella* with affinity for the 6xHis tag in the culture medium supernatant sample ruled out the presence of lysed bacteria as the source of any detected proteins.

Results

Strain VTRS2 is rough and unable to synthesize leucine and grow without complementation

After transformation of *B. suis* 1330 with plasmid pGEM*wbo*A:LCL and removal of the *lox*P cassette with chloramphenicol resistance via pCM158, strain *B. suis* 1330 Δ*wbo*A was confirmed via PCR and sequencing. Following transformation of *B. suis* 1330 Δ*wbo*A with pGEM*leu*B:LCL and subsequent removal of the *lox*P cassette, the resulting strain *B. suis* 1330 Δ*wbo*A Δ*leu*B (VTRS2) was confirmed in the same manner (Figure 2-7). Strain VTRS2 was

then characterized phenotypically to confirm rough morphology and inability to grow without leucine supplementation in the BMM.

Strain VTRS2 was confirmed to have rough colony morphology via crystal violet staining, acriflavine staining, and serum agglutination. The colonies behaved similarly to strain RB51 for all 3 tests; strain VTRS2 takes up crystal violet, clumps in the presence of neutral acriflavine, and fails to agglutinate when incubated with hyperimmune serum against the smooth *Brucella* LPS. Furthermore, the strain was shown to be unable to grow on BMML without complementation of *leu*B on a pNS4-based plasmid. In TSB culture, minimal differences in growth kinetics were observed between the reference strain *B. suis* 1330 and VTRS2 strains (Figure 2-8). All VTRS2 clones remained negative for all antibiotic resistance markers that were used in the intermediate mutagenesis steps (chloramphenicol, ampicillin, and kanamycin).

The candidate immunocontraceptive antigen mGnRHb is expressed by VTRS2-mGnRHb

Plasmid pNS4/mGnRHb was transformed into VTRS2 and colonies which grew on BMML agar were confirmed to contain the plasmid via plasmid extraction (Qiagen), followed by restriction digestion, PCR, and sequencing (Figure 2-9). BamHI and Acc651 were used for restriction enzyme digestion. After confirmation of the presence of the plasmid and insert in the resulting strain VTRS2-mGnRHb, Western blotting was performed to demonstrate expression of the mGnRH antigen. The antigen was detected via anti-HisG-HRP antibody probing using radiography film and colorimetric detection methods (Figures 2-10 and 2-11). Colorimetric detection is a less sensitive method and confirmation via this method is an indicator of strong expression.

The novel fusion antigen RicA-SOD is expressed in VTRS2 but not secreted via the T4SS

Plasmid pNS4/RicA-SOD was generated and transformed into VTRS2 and colonies were selected using BMML agar. Plasmid extraction followed by restriction digestion using BamHI and XbaI, PCR, and sequencing were performed, which confirmed the presence of the plasmid in the resulting strain VTRS2-RicA/SOD (Figure 2-12). Next, Western blot was performed and the ~38 kDa fusion antigen was detected via radiography film and colorimetric detection as described above (Figures 2-13 and 2-14). Then, a supernatant precipitation procedure was performed in triplicate to detect secreted antigen via Western blotting. In all three attempts, the peptide was detected in the bacterial pellet, but never in the supernatant.

Discussion

The candidate vaccine strain VTRS2 was successfully created and shown to have several characteristics desirable of a brucellosis vaccine intended for use in feral swine. Rough colony morphology due to *wbo*A deletion mutation is a known attenuating feature of *Brucella spp.* and is considered the major mutation in the most widely used vaccine strain in the United States, strain RB51 (10). Strain RB51 is derived from *B. abortus*, and has not been shown to be effective against swine brucellosis in controlled studies (7). Despite this, a rough vaccine is essential in countries with brucellosis surveillance programs, as smooth strains interfere with such programs by causing seroconversion in vaccinated animals. Thus, a rough *B. suis* strain makes a logical candidate for an effective vaccine in swine. Deletion mutation of *wbo*A has previously been attempted (30); however, the mutagenesis procedure used involved the permanent insertion of a kanamycin resistance gene, which is undesirable, especially in a vaccine with potential use in wildlife *Brucella* reservoirs such as feral swine. Recently, a rough *B. suis* strain was isolated by

Stoffregen *et al.* (2013) from the urine of a feral pig which has shown promise as a candidate vaccine (31). While promising, the strain is early in its testing and characterization and the exact mutations are unknown. Furthermore, it lacks the *leu*B deletion mutation present in VTRS2, which allows for antigen delivery without the use of antibiotic resistance markers using the pNS4 family of plasmids.

Plasmids containing both *Brucella* and recombinant heterologous antigens can be maintained and their antigens expressed in strain VTRS2. Previously, overexpression of Cu/Zn superoxide dismutase (SODc) has been shown to enhance protection by strain RB51 in the mouse model (9, 27). In this study, SODc was fused to the secretion system effector protein RicA. Though the fusion protein was expressed, it was not secreted as expected. A possible cause of this is that the recombinant protein was too large for the Type 4 secretion system machinery. In a previous study in which RicA translocated a marker peptide, the marker used was much smaller than the SODc fusion molecule (28). Another possible reason for lack of secretion could be that rough strains of *Brucella* have disrupted intracellular trafficking (32). It is possible that some unknown mechanism exists which guides RicA through the type-4 secretion system, which is not activated in rough strains. Lastly, while the promoter *gro*E is less strong than the *trc*D promotor used for expression of the immunocontraceptive antigen mGnRHb, it is still a strong constitutive promoter: this may have led to the recombinant antigen being expressed in inclusion bodies and thus unavailable for export. Nonetheless, this novel antigen merits testing in the mouse model to determine if it can enhance the protective efficacy of strain VTRS2 against virulent *B. suis* challenge.

The recombinant immunocontraceptive peptide mGnRH was modified for expression in *Brucella* (mGnRHb). Following difficulty expressing the original mGnRH in *Brucella*, two

codon usage databases, from the Kazusa DNA Research Institute (kazusa.or.jp/codon) and the GenScript Codon Usage Frequency Table (genscript.com/cgi-bin/tools/codon_freq_table), were used to identify several codons present in the mGnRH transcript which have few to no available corresponding tRNAs in *Brucella* with which to translate them. Upon codon optimization, the synthetic recombinant mGnRHb was transformed into VTRS2 and readily expressed behind the *trcD* promotor on pNS4/*trc*D-*mGnRHb*. Strain VTRS2-mGnRHb could be a useful tool to add to the available resources of wildlife management agencies to combat the growing problem of feral swine over population. Further testing to characterize the strain for its efficacy in protecting against virulent *B. suis* challenge and its ability to cause fertility defects in the mouse model are warranted.

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31. **Stoffregen WC, S. Johnson C, Olsen SC.** 2013. Immunogenicity and safety of a natural rough mutant of *Brucella suis* as a vaccine for swine. Research in Veterinary Science **95:**451-458.
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Chapter 2 Tables and Figures

Table 2-1 – WboA Mutagenesis Primers

Primer:	Sequence:
wboAIf	GGG AAG CTT TCA GCA GGG TCA TCT
wboAIr	GGG GAT ATC TCG TAA GAA CGC AAG
wboAIIf	GGG GAT ATC GGA GTA TGC GGA GCT
wboAIIr	GGG GAA TTC CCT TTA GCC AGC CGG

Table 2-2 – LeuB Mutagenesis Primers

Primer	Sequence
leuBIf	GGGGAATTCAGTTTCGCTCGCGGTGAGTGG
leuBIr	GGGGATATCATGATTTCCTTCGGTTCGCCG
leuBIIf	GGGGATATCTATGCTGGCTGATGCTGGCGG
leuBIIr	GGGAAGCTTTCAGGCCGAAAGTGCCTTGAA

Table 2-3 – VTRS2 Characterization Sequencing Primers

Primer:	Sequence:
wboAIf	GGGAAGCTTTCAGCAGGGTCATCT
wboAIIr	GGGGAATTCCCTTTAGCCAGCCGG
leuBIf	GGGGAATTCAGTTTCGCTCGCGGTGAGTGG
leuBIIr	GGGAAGCTTTCAGGCCGAAAGTGCCTTGAA

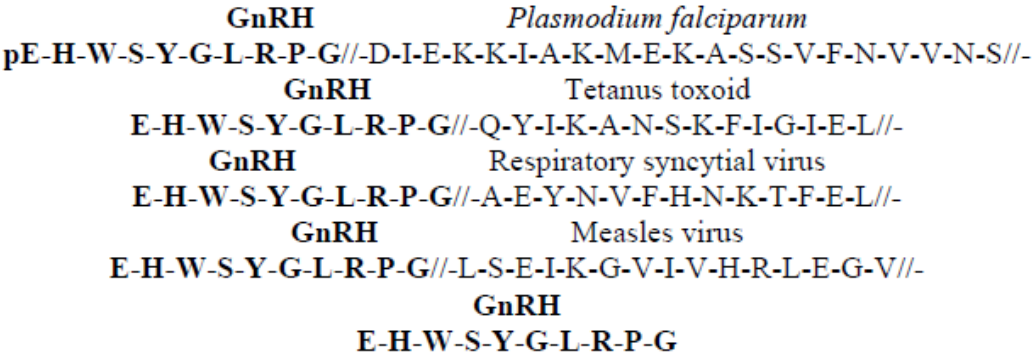
Table 2-4 – Sequencing Primers

Primer:	Sequence:
trcDf seq	GTCGACCAGAAAAAA
pNS4rseq	GGACCGATGCCATCGCCG

Table 2-5 – RicA-SOD Cloning and Sequencing Primers

Primer:	Sequence:
RicAf	GGGggatccATGCCGATCTATGCA
RicAr	GGGggatccATGCCGATCTATGCA
GSG-SODf	GGGagatctGGATCTGGAGAAAGCACGACG
SODr	GGGtctagaCTATTATTCGATCACGCC
pNS4rseq	GGACCGATGCCATCGCCG

Figure 2-1 – The mGnRH “Talwar Recombinant”



Amino acid sequence of the mGnRH immunocontraceptive peptide. "//" Denotes G-S-G linker sequence. From: Miller et al. 2006. Proc 22nd Vertebr Pest Conf. pp106-109 (15)

Figure 2-2 – pGEM*wbo*A:LCL

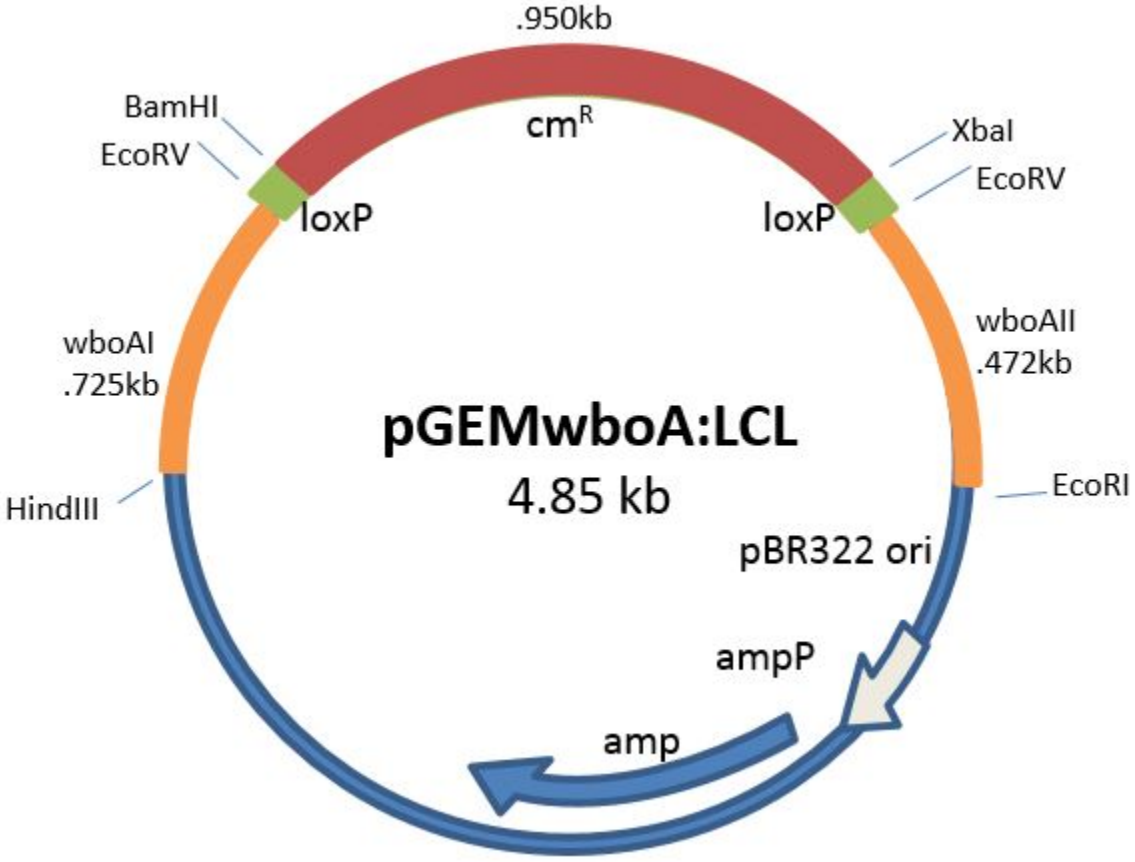


Figure 2-3 – pGEM*leu*B:LCL

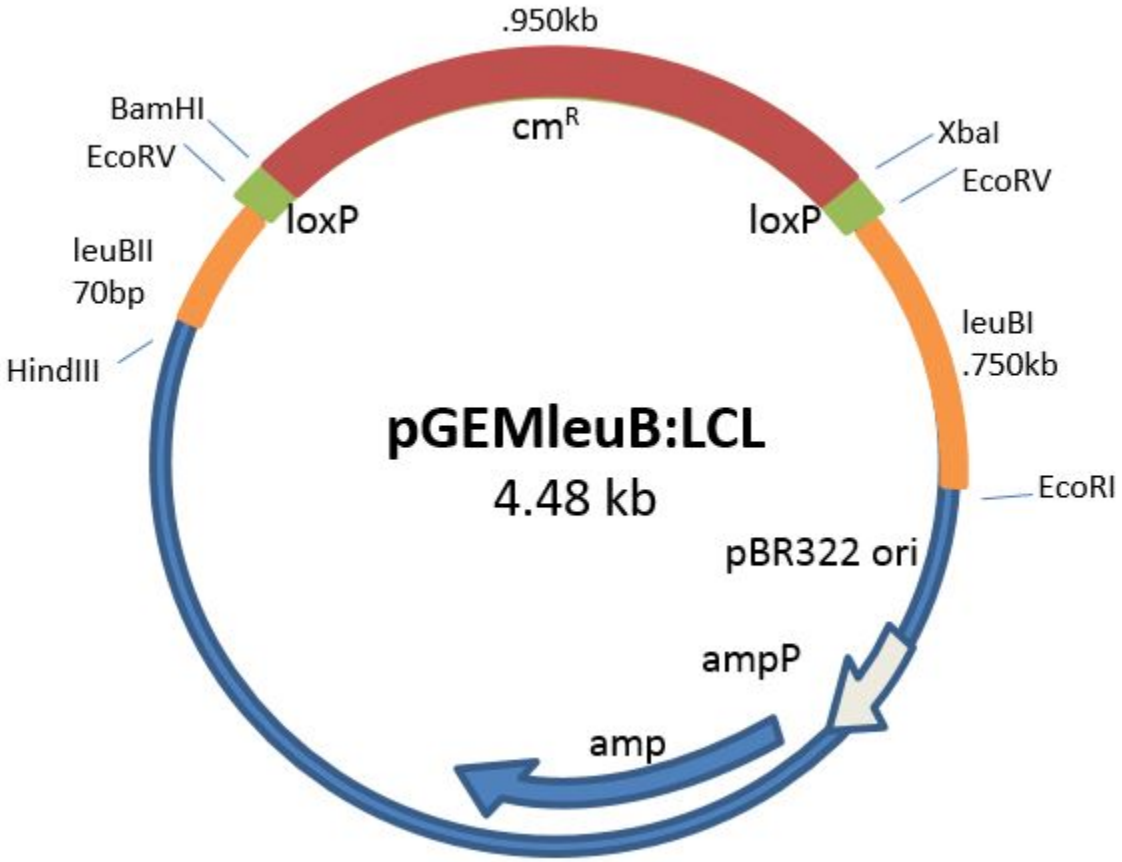
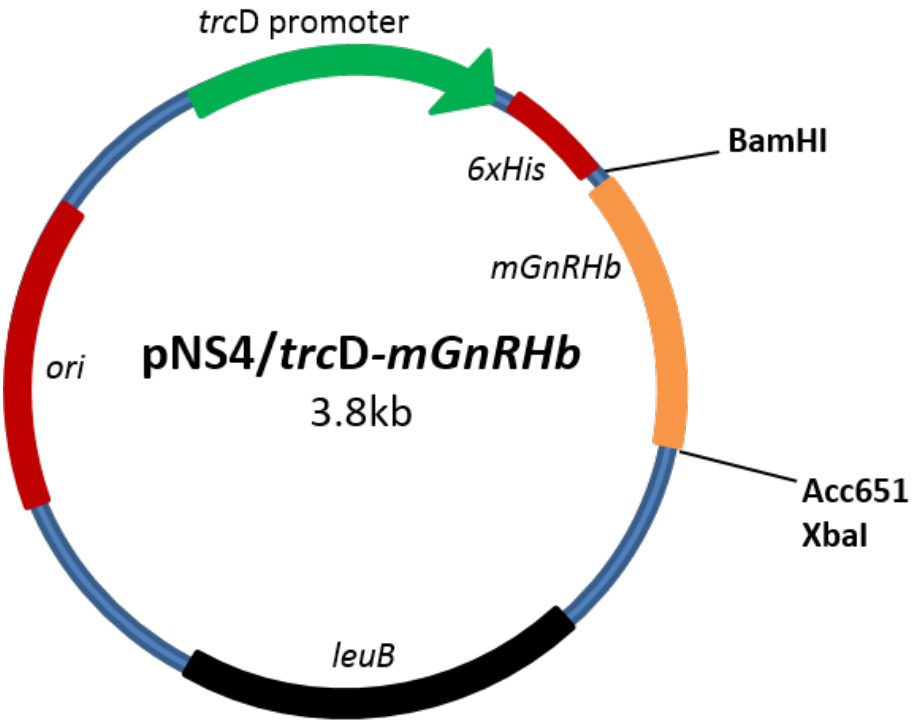


Figure 2-4 – pNS4/*trcD-mGnRHb*



Plasmid pNS4/*trcD-mGnRHb* vector codon optimized for expression of the immunocontraceptive Talwar Recombinant peptide in *Brucella suis*.

Figure 2-5 – 441bp mGnRHb annotated sequence

GGA TCC GAG CAT TGG TCC TAT GGC CTG CGC CCG GGC GGC AGC GGC GAC
BamHI E H W S Y G L R P G G S G D

ATC GAA AAG AAG ATC GCG AAG ATG GAG AAG GCG TCC TCG GTG TTC AAT
I E K K I A K M E K A S S V F N

GTG GTC AAC GGC AAG CTG TCC GGC GAG CAC TGG TCC TAT GGC CTT CGC
V V N G K L S G E H W S Y G L R

CCG GGC TCG GGC GCC GAG TAT AAT GTC TTC CAT AAC AAG ACC TTT GAA
P G S G A E Y N V F H N K T F E

CTG CCG CGC GCA GGC GGC GAA CAC TGG TCC TAC GGT CTG CGC CCG GGT
L P R A G G E H W S Y G L R P G

GGC GGC CAG TAT ATC AAG GCC AAT TCC AAG TTC ATC GGC ATC ACG GAA
G G Q Y I K A N S K F I G I T E

CTG GGC TCC GGC GAA CAT TGG TCG TAT GGC CTC CGC CCG GGC GGC TCG
L G S G E H W S Y G L R P G G S

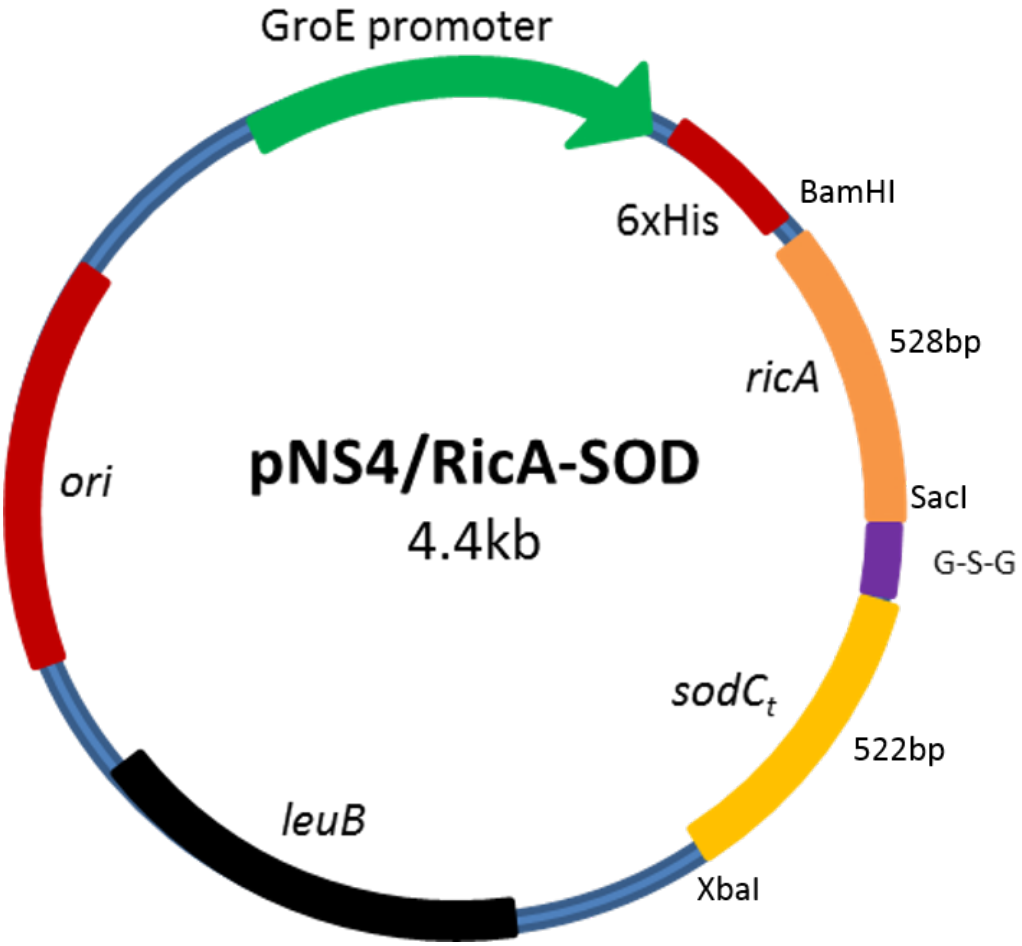
GGC CTT TCC GAA ATC AAG GGC GTT ATC GTG CAC CGC CTG GAG GGC GTT
G L S E I K G V I V H R L E G V

GGC AGC GGC GAA CAT TGG TCC TAT GGC CTG CGC CCG GGC TAA TAA CTC
G S G E H W S Y G L R P G * * XhoI

GAG GGT ACC
KpnI/Acc651

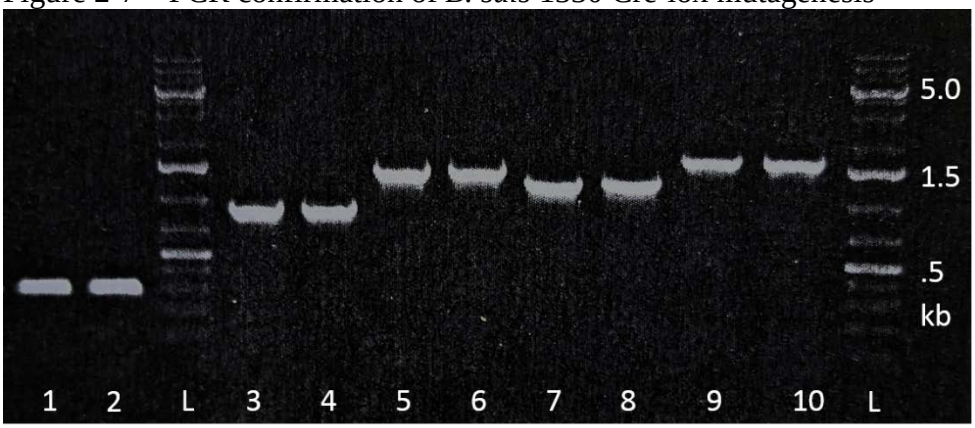
Red = GnRH decapeptide
Black = Flexible linker
Blue = CSP epitope
Green = RSV epitope
Purple = TT epitope
Gray = Measles epitope

Figure 2-6 – pNS4/RicA-SOD



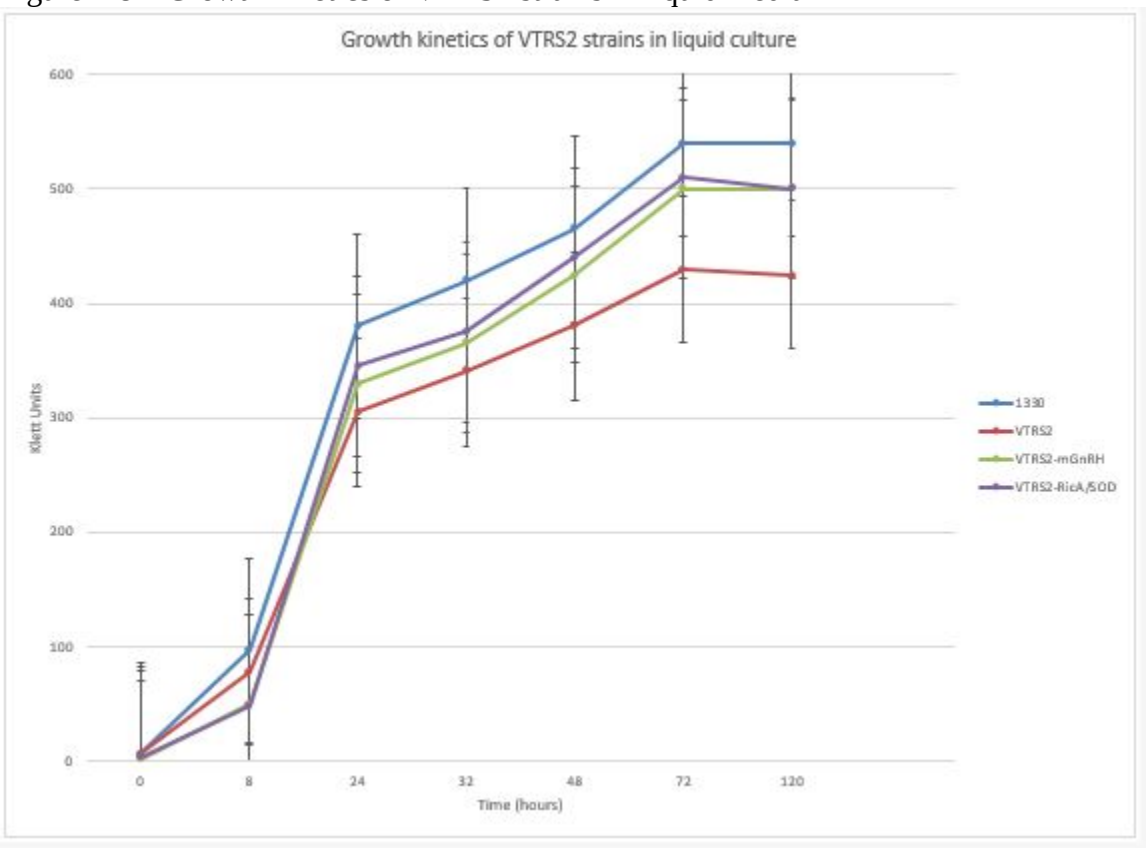
Plasmid pNS4/RicA-SOD containing the native constitutive *Gro*E promoter; *ric*A- Encodes GTPase-interacting protein secreted by the *Brucella* T4SS; *sod*C_t- Encodes the virulence factor Cu/Zn superoxide dismutase lacking the N-terminal periplasmic signal sequence; *leu*B- Encodes isopropyl malate dehydrogenase required for synthesis of leucine

Figure 2-7 – PCR confirmation of *B. suis* 1330 Cre-lox mutagenesis



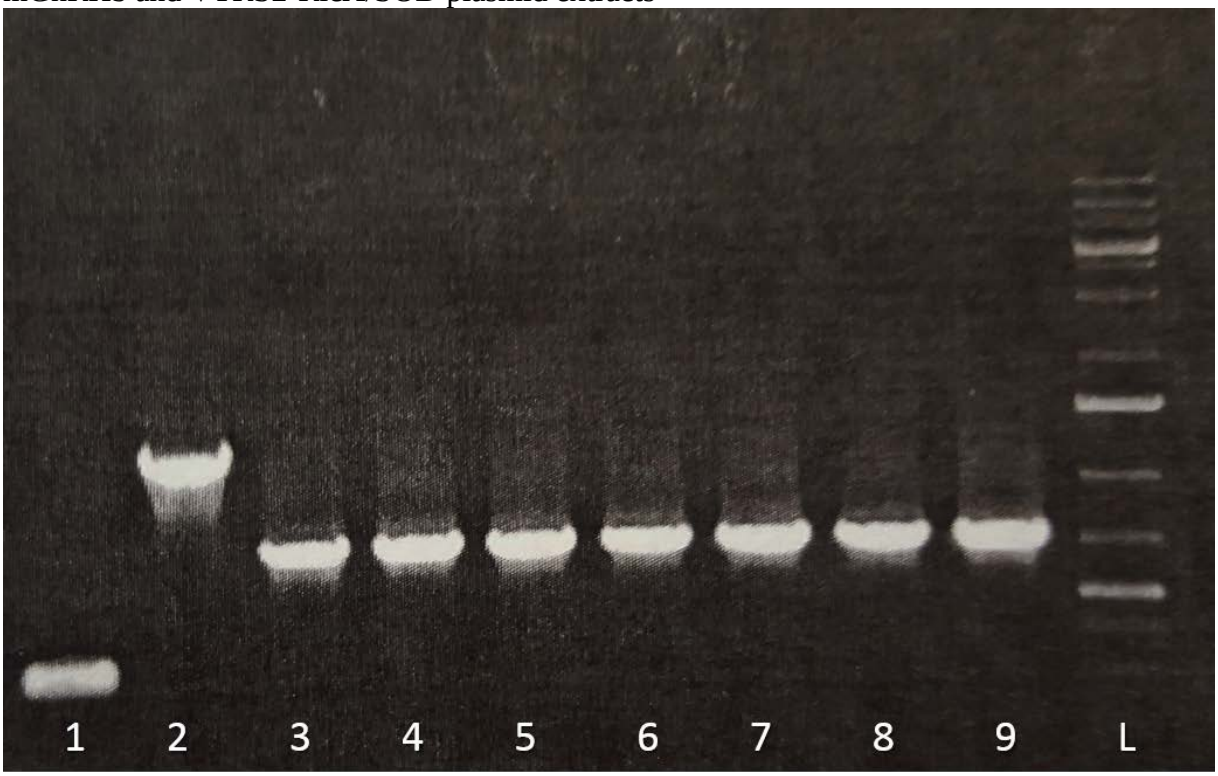
PCR Confirmation of mutagenesis of *B. suis* 1330 to create *B. suis* Δ*wbo*A Δ*leu*B (VTRS2); 1- VTRS2 AMOS PCR, 2- Wild-type *B. suis* 1330 AMOS PCR (WT) 285 bp, L- Fermentas GeneRuler 1kb+ Ladder, 3-4- VTRS2 showing 216 bp *leu*B deletion, 5-6- WT *leu*B, 7-8- VTRS2 showing 410 bp *wbo*A deletion, 9-10- WT *wbo*A; Band size is based on PCR using primer pairs used for mutatgenesis and sequencing, not boundaries of the open reading frame for the respective genes.

Figure 2-8 – Growth kinetics of VTRS2 strains in liquid media



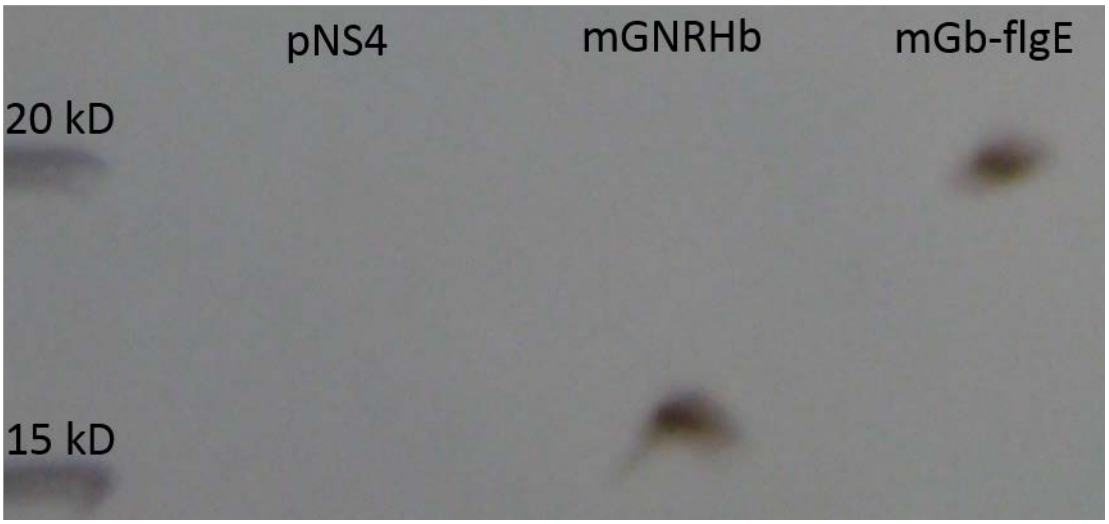
Growth kinetics of *B. suis* 1330, VTRS2, VTRS2-mGnRH, and VTRS2-RicA/SOD in tryptic soy broth. Light scattering was measured in Klett Units. There was no difference in growth among any of the strains as detected by t-test.

Figure 2-9 – PCR confirmation of pNS4/*trc*D-*mGnRHb* and pNS4/RicA-SOD in VTRS2-mGnRHb and VTRS2-RicA/SOD plasmid extracts



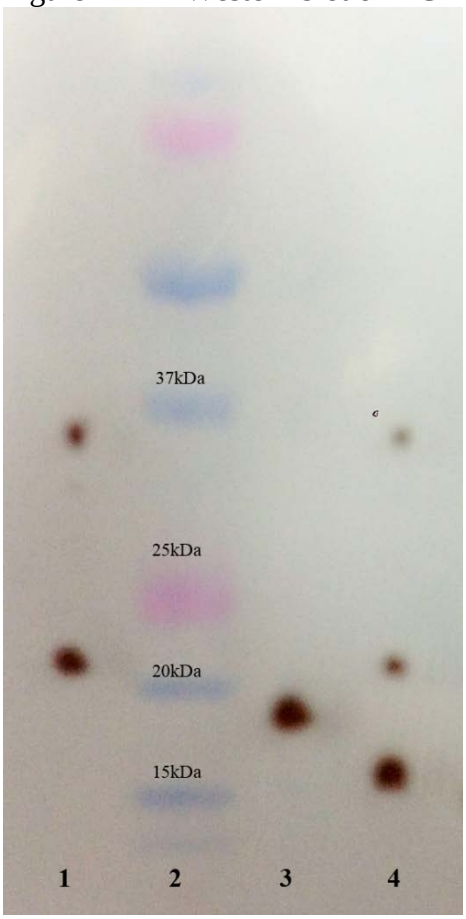
Agarose gel electrophoresis of PCR amplicons from recombinant plasmids pNS4/*trc*D-*mGnRHb* and pNS4/RicA-SOD in VTRS2-mGnRHb and VTRS2-RicA/SOD plasmid extracts. Lanes: 1 – VTRS2 pNS4 with amplification of the multiple cloning site; 2 – VTRS2-RicA/SOD; 3-9 – PCR screened clones of VTRS2-mGnRHb. Bands visualized under ultraviolet light.

Figure 2-10 – Western blot of mGnRHb via light emission detection on radiography film



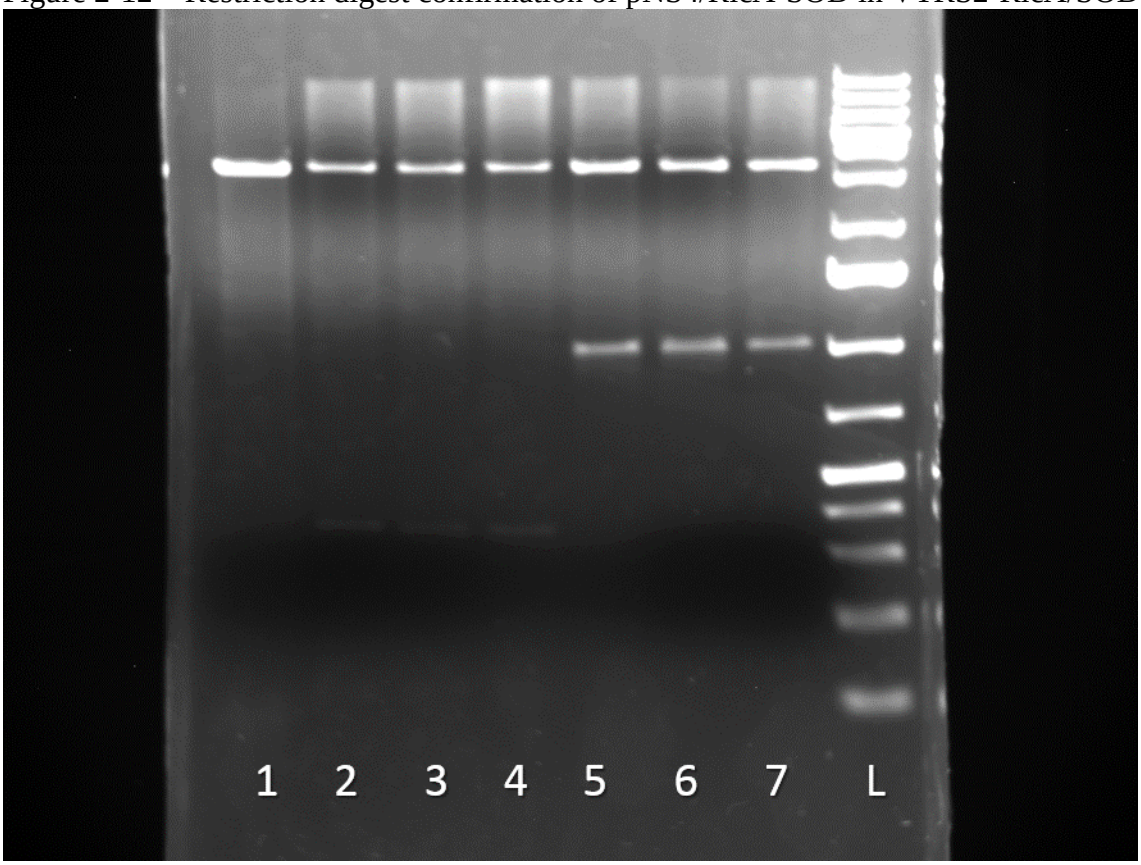
Western blot of extracted protein lysates from VTRS2 with empty expression vector pNS4 (pNS4), VTRS2-mGnRHb with the 16-17 kDa mGnRH antigen (mGNRHb), and VTRS2-flgE-mGnRHb (mGb-flgE) mGnRH antigen with *flg*E periplasmic signal sequence (~19 kDa, see Chapter 5). Anti-HisG-HRP antibody (Invitrogen) was used for generation of light emission detected on radiographic film.

Figure 2-11 – Western blot of mGnRHb via colorimetric detection



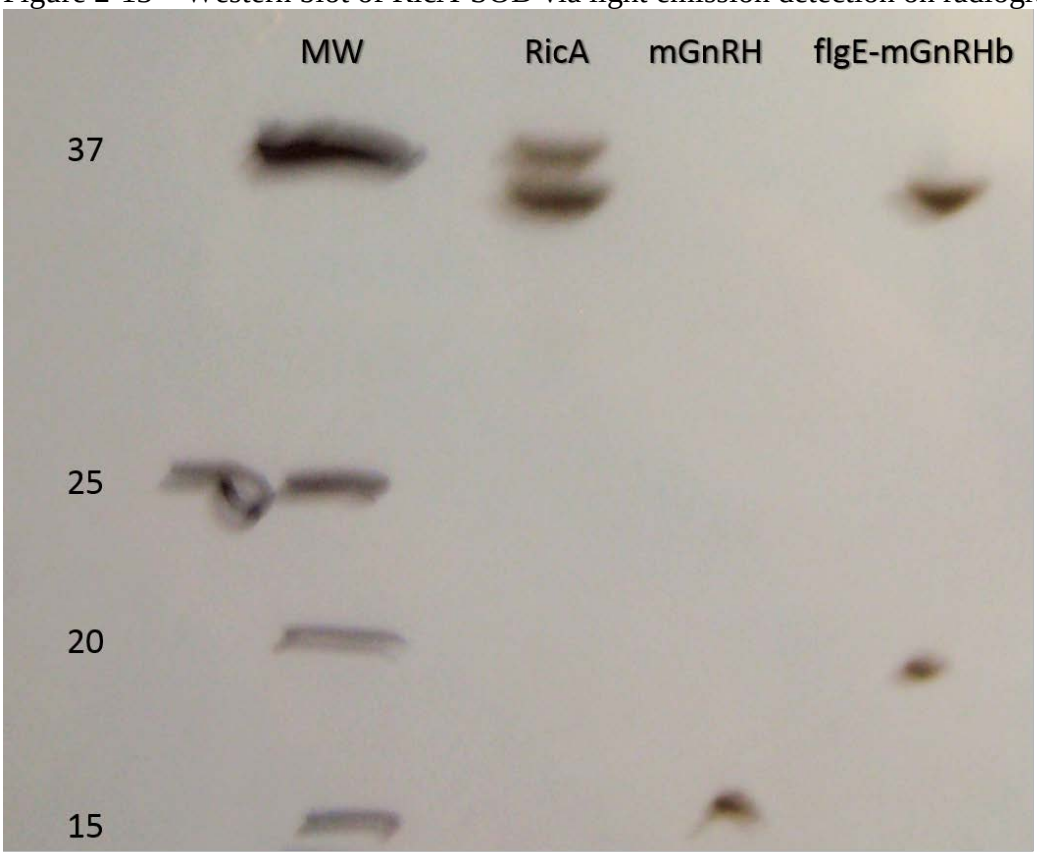
Western blot detection of 6xHis affinity binding to the mGnRHb antigen produced from strain VTRS2-mGnRHb via colorimetric detection. Lanes: 1 – negative control *B. suis* VTRS2 with empty expression vector pNS4, the bands seen are non-specific binding by the Anti-His HRP antibody and are common to *B. suis* protein lysates. 2 – Precision Plus Dual Color Protein Standards (Biorad). 3 – RV2660c-Esat6 19 kDa positive control. 4 – the 17 kDa mGnRH antigen expressed by VTRS2-mGnRHb and non-specific *B. suis* Anti-His affinity protein binding.

Figure 2-12 – Restriction digest confirmation of pNS4/RicA-SOD in VTRS2-RicA/SOD



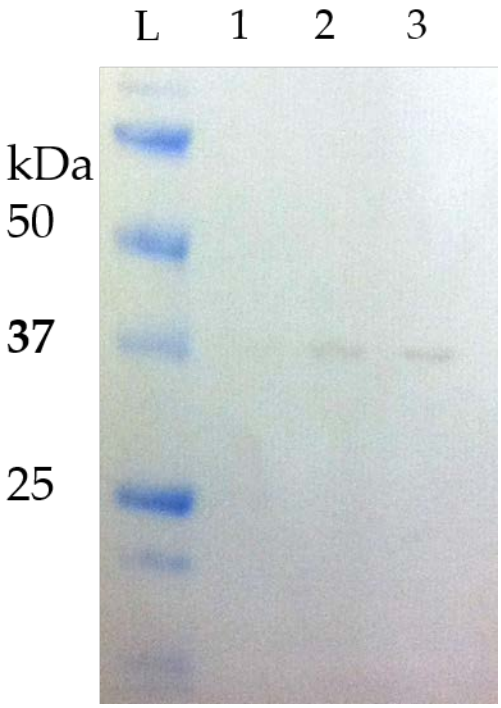
Agarose gel electrophoresis for restriction digest confirmation of pNS4/RicA-SOD in BamHI/XbaI digested VTRS2-RicA/SOD plasmid extract. Lanes: 1 – VTRS2 pNS4, 2-4 – VTRS2-mGNRH, 5-7 – VTRS2-RicA/SOD with 1002 bp RicA-SOD fusion insert, L – 1 kb-plus ladder (Fermentas)

Figure 2-13 – Western blot of RicA-SOD via light emission detection on radiography film



Western blot of RicA-SOD fusion antigen from VTRS2-RicA/SOD protein lysate. Anti-HisG-HRP (Invitrogen) was used for detection via light emission onto radiography film. Lanes: MW – overlay of Precision Plus Protein Dual Color Standards manually annotated onto the radiographic film from the nitrocellulose membrane; RicA – 6xHis tagged RicA-SOD fusion antigen and 36 kDa non-specific his-affinity peptide common to *B. suis* protein lysates; mGnRH – 17 kDa mGnRH positive control antigen; flgE-mGnRHb – ~19 kDa flgE-mGnRHb antigen (see chapter 5) and 36 kDa non-specific his-affinity peptide. While it is expressed behind the *Gro*E promoter, no secretion of the RicA-SOD fusion antigen was detected (not shown).

Figure 2-14 – Western blot of RicA-SOD via colorimetric detection



Western blot colorimetric detection of RicA-SOD expression by VTRS2/RicA-SOD via Anti-His HRP affinity binding. Lanes: L – molecular weight standards; 1 – VTRS2 pNS4 negative control; 2 and 3 – VTRS2/RicA-SOD

CHAPTER 3: *BRUCELLA SUIS* STRAIN VTRS2 IS ATTENUATED *IN VIVO* AND PROTECTS AGAINST VIRULENT *B. SUIS* CHALLENGE

Abstract

Brucellosis is a reproductive disease with a significant negative impact in cattle, swine, and small ruminants and is one of the most prevalent zoonotic diseases worldwide. In the United States, the disease has been eradicated from livestock due to an extensive test and slaughter campaign, continued surveillance, and calf-hood vaccination with the rough *Brucella abortus* strain RB51. Despite this, the disease still exists in wildlife reservoirs, the most abundant being feral swine, and there is no approved brucellosis vaccine for use in domestic or feral pigs. A candidate brucellosis vaccine for use in swine should be live-attenuated, have rough morphology, and protect against virulent *Brucella suis* challenge. *B. suis* strain VTRS2 was developed using cre-lox recombination to create a strain with rough colony morphology, leucine auxotrophy and to make the strain capable of expressing recombinant antigens without the use of antibiotic resistance markers. In this effort, the BALB/c mouse model was used to determine if strain VTRS2 is attenuated *in vivo* and if it can protect against virulent *B. suis* 1330 challenge. It was found that VTRS2, VTRS2 expressing the recombinant antigens RicA/SOD and VTRS2 expressing mGnRHb immunocontraceptive antigen are cleared from female BALB/c mice within 6 weeks post-inoculation. Subsequently, conventional and long vaccination-challenge interval challenge studies were performed to determine if the VTRS2 strains protect against virulent *B. suis* challenge. In the conventional challenge study, all three candidate vaccine strains were protective. In the long-interval study, vaccination with strain VTRS2 and VTRS2 expressing RicA/SOD resulted in a significant reduction in splenic bacterial load versus saline controls.

Strain VTRS2 has the potential to be an effective brucellosis vaccine for use in swine and strain VTRS2-mGnRHb should be evaluated further to assess its ability to cause infertility using the mouse model prior to further testing for use as a tool for feral swine population and disease control.

Introduction

Brucellosis is a zoonotic disease caused by members of the Gram-negative bacterial genus *Brucella*. In humans, the disease causes a severe undulant fever with the potential for chronic arthritis and neurological disease despite aggressive treatment. In animals, brucellosis is mainly a reproductive disease causing abortions, infertility, and subsequent economic losses (1). The main routes of infection to humans are exposure to reproductive secretions, aborted fetuses, placenta, and contaminated milk. Domestic livestock are the traditional reservoirs of *Brucella* and the three major species of the genus have a strong host preference, infecting mainly cattle (*B. abortus*), swine (*B. suis*), and small ruminants (*B. melitensis*) (2). Though the disease has been eradicated from domestic cattle and swine in the United States, *B. abortus* and *B. suis* remain in wildlife reservoirs including elk (*Cervus elaphus*), bison (*Bison bison*), and feral swine (*Sus scrofa*), which continue to pose the threat of reintroduction of the disease into domestic species as well as humans (3).

Among the wildlife reservoirs of brucellosis in the United States, feral swine are the most widespread, and they have been implicated as the cause of infections in both livestock and humans (4, 5). Furthermore, the disease continues to exist in domestic swine herds worldwide and there is no widely approved vaccine against *B. suis* infection in swine (6). In cattle, the official vaccine strain in the United States is the rough attenuated strain *B. abortus* RB51.

Though RB51 does not appear to be effective in pigs, it has several traits that are desirable of a brucellosis vaccine for use in swine. The rough mutation, which attenuates the strain, also allows vaccinated animals to be differentiated from infected animals (DIVA) which is of major benefit to effective surveillance and control programs (7). Furthermore, RB51 is a modified-live vaccine and therefore is consistently more effective in protecting against virulent *Brucella* challenge than other formulations such as bacterins (8). However, RB51 has several disadvantages that make it less-than ideal for use in feral swine. In addition to being poorly effective in swine in controlled studies, another disadvantage of RB51 is that it carries rifampin resistance. This problem is two-fold; first, rifampin is a recommended antibiotic for prophylaxis and treatment in cases of human *Brucella* exposure (9). Second, a vaccine with potential for use in wildlife species such as feral swine should not carry with it the risk of introduction of antibiotic resistance genes into the environment.

Previously, mutagenesis has been performed in *Brucella* using the cre-lox system of recombination, which leaves no permanent antibiotic resistance marker in the resulting mutant strain (10, 11). This technology was used to create strain *B. suis* 1330 Δ*wbo*A Δ*leu*B, called VTRS2, which is a rough mutant and a leucine auxotroph of *B. suis* capable of expressing recombinant antigens on the pNS4 family of plasmids without the need for antibiotic resistant markers for selection. In this work, strain VTRS2 was characterized in the mouse model to establish whether the strain is sufficiently attenuated *in vivo* for use as a candidate vaccine and to demonstrate if the vaccine can protect mice against virulent *B. suis* challenge. In addition to VTRS2, VTRS2 strains expressing either the recombinant *Brucella* antigen RicA/SOD or the immunocontraceptive peptide mGnRH (12) were also tested to determine their ability to clear from the host and protect against virulent challenge. Challenge studies were performed, both

according to the conventional model used in brucellosis vaccine development as well as using a longer vaccination-to-challenge interval, to evaluate if the strains are capable of protection if exposure to virulent organisms occurs later than is usually assessed.

Materials and Methods

Vaccine strains

Virulent *B. suis* 1330 was used as the wild-type control in the clearance study and as the challenge strain in the protection studies. The candidate vaccine strains used consist of the defined mutant *B. suis* 1330 Δ*wbo*A Δ*leu*B (VTRS2) as the platform to maintain one of three plasmids: empty expression vector (pNS4), pNS4 carrying the recombinant immunocontraceptive antigen mGnRHb (pNS4/*trc*D-*mGnRHb*) behind the synthetic promoter *trcD*, and pNS4 carrying the recombinant *Brucella* antigen RicA/SOD (pNS4-RicA/SOD) behind the native constitutive promoter *groE*. Strain VTRS2 was generated using cre-lox recombination technology to generate defined deletion mutations using homologous recombination without leaving a permanent antibiotic resistance marker in the chromosome. VTRS2 was transformed with the expression vectors via electroporation and expression of the recombinant antigens confirmed via Western blot prior to inoculation of mice. All manipulations of live *Brucella* were performed in a CDC-approved biosafety level 3 (BSL-3) facility.

Animal use

All animals used were female BALB/c mice (Harlan Laboratories) between 4 and 6 weeks of age. Mice were housed in a CDC approved animal BSL-3 facility and cared for by the VMCVM Teaching and Research Animal Care and Support Staff (TRACSS). The Institutional Animal Care and Use Committee approved all experiments and procedures. Euthanasia was

performed via CO_2 asphyxiation followed by cervical dislocation. Any blood collections were done via retro-orbital plexus venipuncture under isoflurane anesthesia to ensure collection of an adequate volume of plasma.

Clearance study

To evaluate the attenuation of the VTRS2 candidate vaccine strains, four to six week old female BALB/c mice were inoculated intraperitoneally (IP) with approximately $5x10^5$ CFU (colony-forming units) of *B. suis* 1330, VTRS2 pNS4 (expression plasmid without insert), VTRS2 RicA-SOD or VTRS2-mGnRHb. Each VTRS2 group of mice was euthanized and processed at weeks four and six (n=5 per time point). Additional *B. suis* 1330 groups were similarly processed at eight and ten weeks (n=5). Immediately following euthanasia, splenic CFU were determined by plating serially diluted spleen homogenates on tryptic soy agar (TSA). Acceptable clearance for live vaccine efficacy was considered between four and eight weeks.

Challenge study

For the challenge study, following the conventional model for brucellosis vaccines, 4 to 6 week old female BALB/c mice were administered ~5×10^5 CFU IP of either VTRS2/pNS4, VTRS2/mGnRHb, or VTRS2/RicA-SOD (n=10), and an additional group was administered sterile saline. At six weeks post-vaccination, all mice were either boosted with 5×10^4 CFU or challenged with 5×10^4 *B. suis* 1330 (n=5). All mice were euthanized two weeks post-challenge and splenic CFU determined by serial dilution.

Long vaccination-challenge interval study

For the late exposure study, forty mice, were vaccinated with either VTRS2/pNS4, VTRS2/mGnRHb, or VTRS2/RicA-SOD as before. Each group was then either boosted with ~5×10^4 CFU IP of the vaccine strain or challenged with 4×10^4 *B. suis* 1330 eight weeks after

initial vaccination (n=5). Boosted animals were challenged four weeks post-booster. All animals were euthanized two weeks post-challenge and splenic CFU determined. Mice in the boosted groups were also bled retro-orbitally at four and six weeks, plus two weeks following a booster dose of $5x10^4$ CFU and plasma collected to determine anti-mGnRH antibody response.

Anti-mGnRH ELISA

The mGnRH antibody response using purified mGnRH was assessed via enzyme-linked immunosorbent assay (ELISA). Purified mGnRH was obtained from pRSETb-mGnRH grown in *E. coli* DH10b and purified using Ni-His affinity chromatography (Qiagen). Plasma was diluted 1:100 and each well was coated with 1.0μg of the purified antigen. After primary incubation, plates were washed and incubated with 1:2000 horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Invitrogen). Absorbance was measured on a microtiter plate reader at 450nm. Samples from mice inoculated with VTRS2-mGnRHb were compared with those receiving VTRS2 with empty plasmid as well as pre-inoculation samples. Absorbance values were compared via 2-way ANOVA.

Statistical analysis

Splenic titers were compared using Student's t-test to compare means between individual groups. All statistical analysis was performed using the JMP Pro 11 software (SAS).

Results

VTRS2 is attenuated in mice

At four weeks post-inoculation, all VTRS2-inoculated mice had between 2.3 and 2.6 LOG reduction (>97%) in splenic CFU versus control mice infected with virulent *B. suis* 1330 ($P<0.001$, Figure 3-1). At six weeks, all VTRS2 inoculated mice had >3.8 LOG reduction

(>99.98%) in splenic CFU versus controls and had reached the minimum limit of detection (1.3 LOG) and were considered cleared. There was no difference in CFU between samples plated on enriched agar versus leucine-deficient agar suggesting the pNS4 plasmid was maintained *in vivo*. This agrees with previous findings (H. Alqublan, Virginia Tech, unpublished research).

VTRS2 protects mice against challenge

In the conventional challenge study, mice were either challenged or boosted six weeks post-vaccination. In the single-dose groups, 0.73, 0.47, and 0.64 LOG reduction in CFU were observed for mice given VTRS2/RicA-SOD, VTRS2/mGnRHb, and VTRS2 pNS4, respectively (all reductions significant, $P<0.05$) versus unvaccinated controls (Figure 3-2). After booster, LOG reduction in mice receiving VTRS2 RicA-SOD was 1.05 ($P<0.001$), 0.6 for VTRS2-mGnRHb ($P<0.05$), and 1.25 for VTRS2 pNS4 ($P<0.001$). All groups were significantly different from unvaccinated controls and VTRS2 with the control plasmid pNS4 was significantly more protective than VTRS2-mGnRHb ($P<0.01$) (Figure 3-3).

In the long post-vaccination interval study, the VTRS2/mGnRHb and RicA-SOD vaccinated groups experienced approximately 0.5 LOG reduction (~60%) in splenic CFU without a booster and were significantly different from the saline control. The pNS4 empty plasmid group experienced 0.23 LOG reduction and was not significantly protective (Figure 3-4). In the groups receiving a booster vaccination at eight weeks post-vaccination, only the RicA-SOD group was significantly different from the controls (0.69 LOG reduction in splenic CFU, $P=0.0146$) (Figure 3-5).

Strain VTRS2-mGnRHb elicits a significant anti-mGnRH immune response

Peak mGnRH-specific antibody titers occurred at four weeks post-vaccination and plasma titers were significantly ($P \leq 0.05$) higher in VTRS2-mGnRHb vaccinated versus VTRS2

pNS4 vaccinated mice at four and six weeks post-vaccination. Titers two weeks after booster vaccination were elevated in both groups but there was no difference between the mGnRH and control pNS4 groups (Figure 3-6).

Discussion

The *wboA* mutation in strain VTRS2 was hypothesized to attenuate the strain such that it cleared from BALB/c mice within eight weeks. A previous experiment with a *wboA* mutation in *B. suis* demonstrated clearance in six to eight weeks (13). Clearance between four and eight weeks is desirable for a live *Brucella* vaccine to ensure adequate cell-mediated immunity is generated without allowing an unacceptable infection to become established from the vaccine strain. The data show that strain VTRS2 is attenuated *in vivo* and evaluation of the vaccine strains for protection against challenge was pursued accordingly. There was no difference in titer between splenic samples plated on TSA versus *Brucella* Minimal Media deficient in leucine. This demonstrates the stability of the pNS4 family of plasmids *in vivo*. Furthermore, presence of the antigen-delivery plasmids pNS4/mGnRHb and pNS4/RicA-SOD did not affect clearance kinetics of VTRS2 in mice.

Strain VTRS2-mGnRH was able to elicit a significant IgG immune response against the mGnRH antigen at four and six weeks post-inoculation. For the mGnRH immunocontraceptive to cause a fertility defect in the host, a significant antibody response against endogenous gonadotropin releasing hormone (GnRH) must be raised. Future studies are warranted to assess this ability. Interestingly, though the non-specific level of IgG was increased in both the VTRS2 pNS4 control and VTRS2/mGnRHb after booster vaccination, there was no significant difference between the two. This is most likely due to non-specific binding resulting from elevated antibody

levels in response to other vaccine antigens. It also could be a result of rapid clearance of the vaccine strain upon booster vaccination before the strain has sufficient time to express enough mGnRH antigen to elicit a specific anamnestic response against it. Nonetheless, the clearance and immunology data suggest that fertility studies to further characterize the VTRS2/mGnRHb strain are warranted in addition to the challenge studies.

In the long-interval challenge study, protection was generally poor. These results were not especially surprising given how much time elapsed between initial vaccination and challenge. In the boosted group, only the RicA/SOD group was significantly protected. It is difficult to compare the boosted with non-boosted groups as the challenge dose was higher by approximately 0.5 LOG in the boosted group; however, the results were surprising in that the boosted groups did not significantly protect. This could be attributed to the long interval between vaccination and booster, long interval between initial vaccination and challenge (ten weeks in the boosted group), low booster dose, higher challenge dose in the boosted group, or the age of the animals at time of euthanasia. The long-interval challenge study allowed for the best utilization of surplus mice available following the clearance study and also gives an indication of how apparent vaccine efficacy decreases with deviation from the conventional model. This deviation is relevant given the potential for variability in dose and timing of delivery of vaccine in a wildlife setting.

With both single-administration and in the boosted groups, vaccination of mice with VTRS2 expressing the recombinant *Brucella* fusion antigen RicA/SOD resulted in significant reduction in splenic burden after challenge versus saline controls where VTRS2 carrying the empty plasmid pNS4 did not. However, the difference between the RicA/SOD group and the pNS4 group was not significant and the trend was not evident in the conventional challenge

study, so it cannot be concluded that expression of RicA/SOD significantly enhanced protection in the mouse model. It is, however, significant that some protection was achieved despite the long period of time that elapsed between vaccination and challenge.

In the conventional challenge study, the established murine model for determination of protection in brucellosis vaccine research was used. In both the single-dose and boosted groups, all of the tested strains were significantly protected against virulent *B. suis* challenge. In the groups receiving a single administration of VTRS2, protection was not as strong as predicted. However, while protection was not as high as expected, it was similar to what has been reported by vaccination with a 3 LOG higher dose of strain RB51 (13, 14). Interestingly, the vaccine strain carrying the control plasmid pNS4 was significantly more protective than the immunocontraceptive strain VTRS2-mGnRHb in the boosted group (P <0.01). A possible reason for this is the high metabolic demand placed on the bacteria by the strong *trcD* promotor and possible formation of inclusion bodies caused by high concentrations of mGnRH, leading to a toxic effect on the organism. As in the long-interval study, expression of RicA/SOD did not confer additional protection over VTRS2 expressing empty plasmid. It is possible that an effect could be seen if the antigen were placed behind the stronger *trcD* promoter rather than the native *groE* promoter used in the study, though antigens expressed behind the *groE* promoter have been able to achieve enhanced protection previously (15). The lack of response behind *groE*, a moderately strong promoter (16), makes it unlikely that a significant benefit would be achieved using *trcD* for homologous antigen in this case.

From these studies, it can be concluded with confidence that VTRS2 strains of *B. suis* are attenuated *in vivo* and can be made to stably maintain the pNS4 family of plasmids for up to six weeks. Using VTRS2 as a platform strain, homologous and heterologous antigens could be

expressed; however, neither antigen used in the challenge studies was able to enhance protection against experimental *B. suis* infection versus VTRS2 alone, though all strains conferred significant protection when compared to saline-vaccinated controls. The least protective strain, VTRS2/mGnRHb, still conferred over 70% reduction in splenic bacterial load versus controls. This fact, combined with the significant IgG humoral immune response raised against the antigen upon single administration, merits further investigation of the immunocontraceptive strain for its potential to elicit an infertility effect in the mouse model. Furthermore, strain VTRS2 has been demonstrated to decrease systemic dissemination by virulent *B. suis* in experimentally infected feral swine (See Addendum 1). These findings support the overall hypothesis that strain VTRS2 may serve as a useful tool for disease and population control in feral swine, the last major reservoir of *Brucella suis* in the United States and a major reservoir for the pathogen worldwide.

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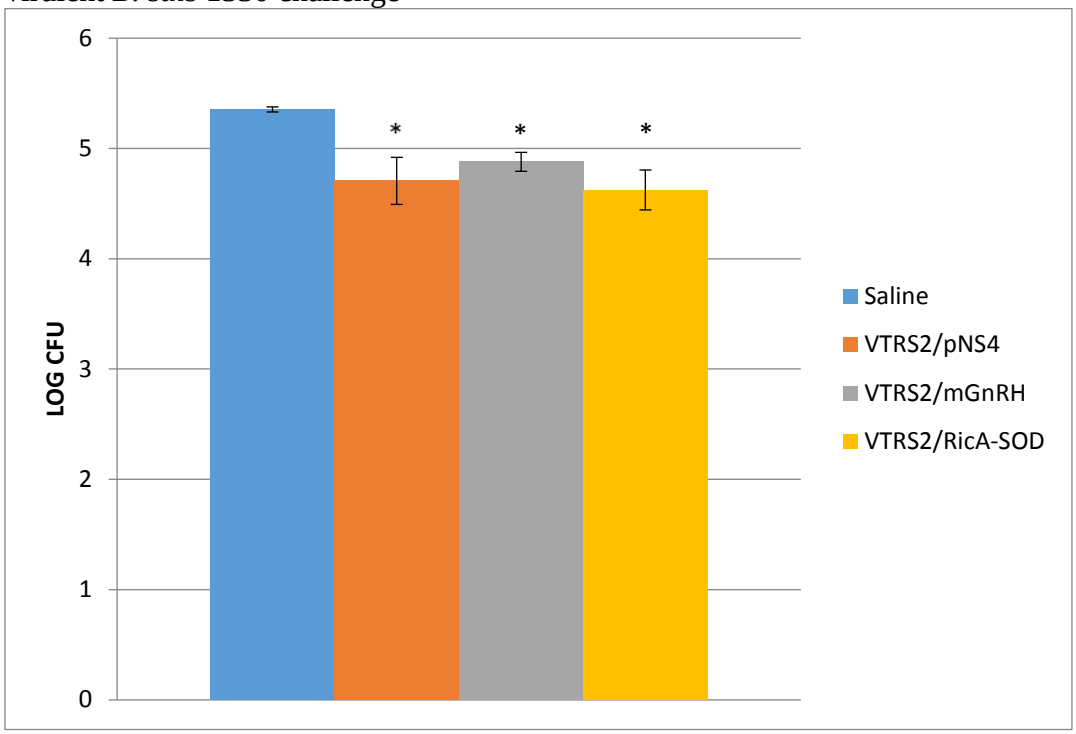
Chapter 3 Figures

Figure 3-1: Clearance kinetics of VTRS2 from BALB/c mice

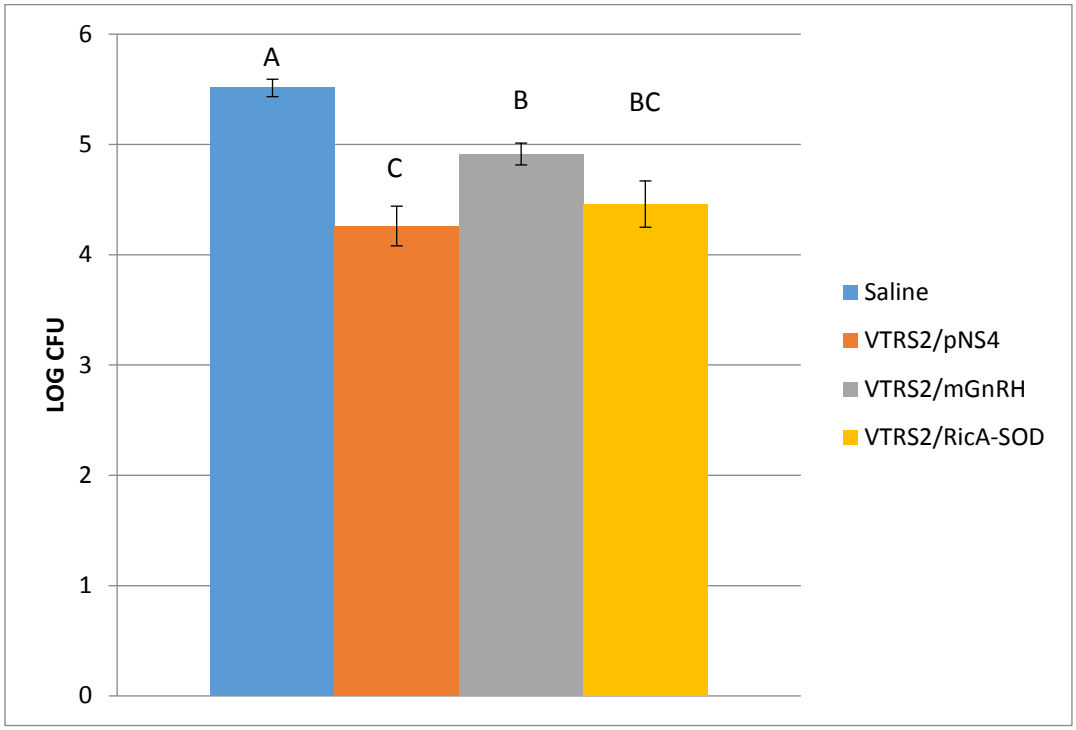
Splenic titers in all vaccinated mice were significantly lower than the virulent strain control mice at all time-points measured and all vaccinated mice reached the lower limit of detection (20 CFU/ml of splenic homogenate, 1.30LOG) by 6 weeks post-inoculation. All mice received an initial dose of ~5 x 10^5 CFU. Titers are reflected as LOG CFU/ml of Splenic homogenate.

Figure 3-2: Mice receiving a single administration of *B. suis* VTRS2 are protected against virulent *B. suis* 1330 challenge



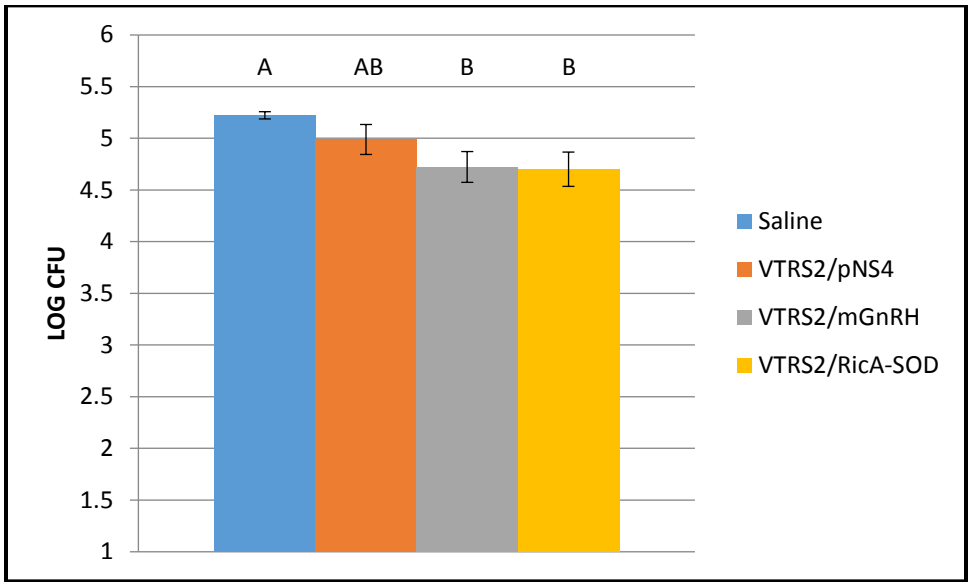
LOG CFU/ml of splenic homogenates in single-vaccinated mice 2 weeks after virulent *B. suis* 1330 challenge in mice vaccinated with VTRS2/pNS4, VTRS2/mGnRHb, VTRS2/RicA/SOD, or sterile saline. * = $P < 0.05$

Figure 3-3: Splenic bacterial load in vaccinated and boosted mice challenged 8 weeks after initial vaccination



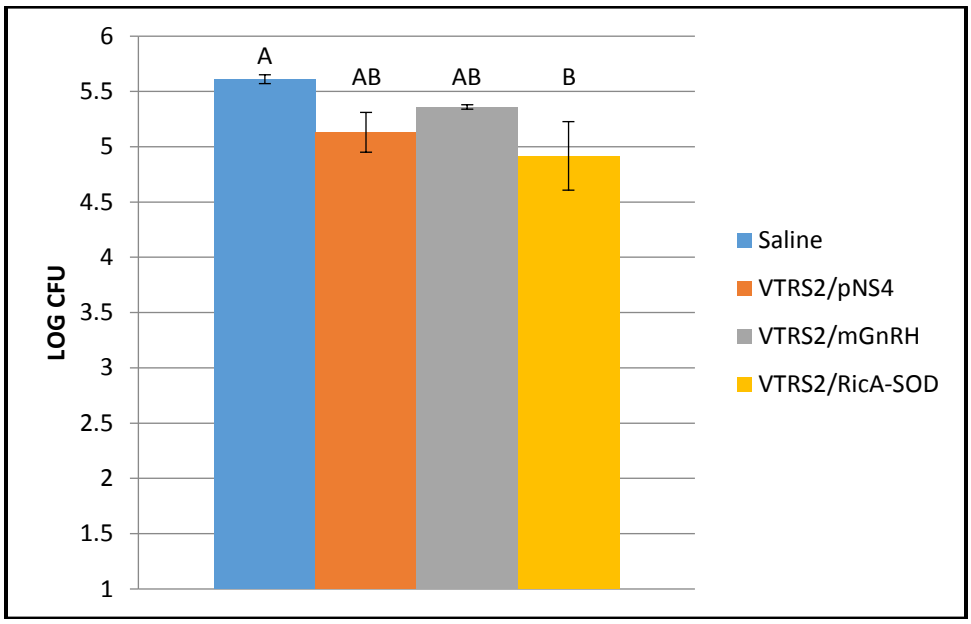
Splenic titer of *B. suis* 1330 challenge strain two weeks post-challenge in mice vaccinated with VTRS2/pNS4, VTRS2/mGnRHb, VTRS2/RicA/SOD, or sterile saline that received a booster vaccination six weeks after initial inoculation. Different connecting letters signify statistical significance ($P < 0.05$).

Figure 3-4: VTRS2 expressing the immunocontraceptive mGnRH or the fusion antigen RicA/SOD significantly protects mice against virulent *B. suis* challenge 8 weeks after a single vaccination dose



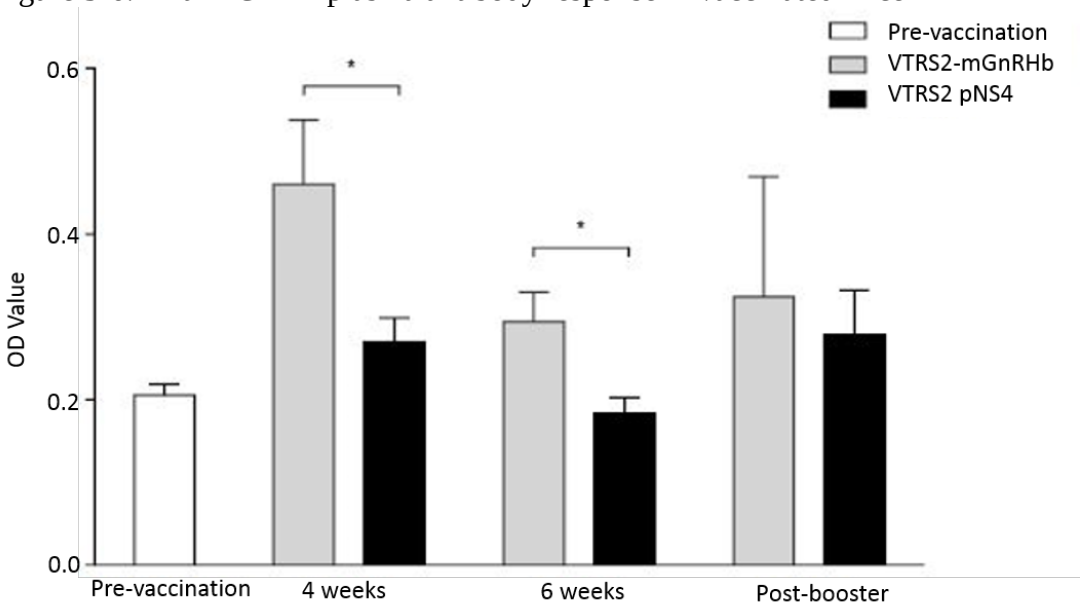
Splenic titer of *B. suis* 1330 challenge strain two weeks post-challenge in mice vaccinated with VTRS2/pNS4, VTRS2/mGnRHb, VTRS2/RicA/SOD, or sterile saline eight weeks prior to challenge. Connecting letters signify statistical significance. The mGnRH and RicA/SOD expressing strains were significantly protective ($P < 0.05$) while VTRS2 carrying empty expression vector was not. Different connecting letters signify statistical significance.

Figure 3-5: Splenic bacterial load in vaccinated and boosted mice challenged 12 weeks after initial vaccination



Splenic titer of *B. suis* 1330 challenge strain two weeks post-challenge in mice vaccinated with VTRS2/pNS4, VTRS2/mGnRHb, VTRS2/RicA/SOD, or sterile saline that received a booster vaccination eight weeks after initial inoculation. Challenge occurred 4 weeks after booster (twelve weeks post initial vaccination). Mice receiving VTRS2 expressing the fusion antigen RicA/SOD were significantly protected against virulent *B. suis* challenge ($P < 0.05$). Connecting letters signify statistical significance.

Figure 3-6: Anti-mGnRH plasma antibody response in vaccinated mice



Mouse anti-mGnRH IgG Response at plasma dilution 1:100 at 0, 4, and 6 weeks post-vaccination and 2 weeks following booster vaccination. The coating-antigen used was purified mGnRH peptide and antibody binding was detected using peroxidase-conjugated anti-mouse IgG. VTRS2 – VTRS2 with empty expression vector pNS4; VTRS2/GnRH – VTRS2 expressing the mGnRH antigen on pNS4/*trc*D-*mGnRHb*. * = $P < 0.05$

CHAPTER 4: EVALUATION OF THE EFFECT OF VTRS2/MGNRHB ON FERTILITY IN THE MOUSE MODEL AS A CANDIDATE IMMUNOCONTRACEPTIVE VACCINE FOR USE IN FERAL SWINE

Abstract

Immunocontraceptive vaccines have been explored as tools for wildlife population control; however, most formulations are subunit vaccines, which are expensive to produce, require potent adjuvants, and parenteral delivery, which can lead to severe injection site reactions. One species for which immunocontraceptives have been proposed for population control is feral swine (*Sus scrofa*). Feral swine are one of the major nuisance invasive species in the United States and cause upwards of $1.5 billion annually in property, agricultural, and economic losses. Furthermore, feral swine can serve as reservoirs for zoonotic diseases with significant public health and agricultural importance. As their population continues to expand, further methods of control are needed to help wildlife managers mitigate their spread. Delivery of immunocontraceptive antigen using a live attenuated bacterial vaccine platform could address this problem without the high cost of subunit antigen delivery. This study sought to evaluate *Brucella suis* strain VTRS2 expressing multiple gonadotropin releasing hormone (mGNRHb) for its ability to cause reproductive deficits in the mouse model. A 2x2 breeding study design was used to determine if vaccination of males or females could cause a decrease in fecundity or pregnancy rate. It was found that while VTRS2/mGnRH is able to generate an anti-mGnRH immune response, fertility was not compromised compared to unvaccinated controls used in the study.

Introduction

Feral swine (*Sus scrofa*) are the second most costly mammalian nuisance and invasive species annually in the United States, behind only rats and mice in agricultural and property damage (1). These damages are estimated to be around $1.5 billion dollars per year and do not include the cost to landowners, producers, and wildlife management agencies in controlling the population. Feral swine have increased in both range and number; their population has quadrupled to over four million animals in the past decade and they are now found in at least 38 states (2). This increase in population has led to an increased risk of transmission of diseases such as brucellosis, influenza, leptospirosis and pseudorabies to both humans and domestic livestock. Brucellosis, caused by members of the Gram negative bacterial genus *Brucella,* is a major zoonotic disease worldwide which has been eradicated from domestic swine and cattle in the United States (3). Feral swine are the most widespread remaining reservoir of this disease in the U.S. and have been responsible for recent transmission to humans (4, 5). The reintroduction of the disease into domestic herds could have severe economic consequences for U.S. agriculture, so controlling the population of feral swine is therefore important not only to reduce the damage they currently cause, but also to reduce their risk to agriculture and public health. The current predominant methods for control, hunting and trapping, have proven ineffective on their own and are not suited for every situation (such as urban settings), and more control methods are needed (6). One such method which has been investigated is the use of immunocontraceptive vaccines (7). Immunocontraceptive infertility agents rely on the administration of an antigenic form of an endogenous reproductive hormone and subsequent generation of a host immune response against the exogenous antigen and endogenous hormone. Among these, gonadotropin-releasing hormone (GnRH), appears the most promising (8). Anti-

GnRH eliciting vaccines such as GonaCon, a subunit preparation of GnRH linked to the antigenic carrier molecule keyhole limpet hemocyanin, have been previously tested in feral swine with positive results (9, 10). There are several disadvantages of subunit vaccines including cost of production, injection site reactions, and parenteral delivery (9, 11, 12). As an alternative, this study sought to evaluate the efficacy of a recombinant form of GnRH, mGnRHb, delivered in the live-attenuated *B. suis* vaccine VTRS2. The recombinant antigen, mGnRH, is a multimeric form of GnRH decapeptide units alternating with known antigenic B and T-cell epitopes (12, 13) which has been codon-optimized for expression in *Brucella* (mGnRHb). Strain VTRS2/mGnRHb has been previously shown to protect against *B. suis* challenge in the mouse model and to elicit an mGnRH antibody response against the recombinant antigen (see chapter 3). To evaluate the potential for the vaccine to produce an infertility response, the mouse model was used in a pilot breeding study to assess the effect of the VTRS2/mGnRHb vaccine on reproduction in BALB/c mice.

Materials and Methods

Vaccine strains

All manipulations of live *Brucella* were performed in a CDC approved biosafety level 3 (BSL-3) facility.

Animal use

All animals used were BALB/c mice (Harlan Laboratories) approximately 9 weeks of age at the start of the study (post-pubertal). Mice were housed in a CDC-approved Animal BSL-3 facility and cared for by the VMCVM Teaching and Research Animal Care and Support Staff (TRACSS). All mouse experiments and procedures were approved by the Institutional Animal

Care and Use Committee. Euthanasia was performed via CO_2 asphyxiation followed by cervical dislocation.

Study design

To evaluate the interactions between vaccinated and unvaccinated males and females, 42 BALB/c mice (36 females and 6 males, divided into groups of 3 females per male) were used in two rounds of breeding in a 2 x 2 table study design (Table 4-1). At approximately 2 months of age, 18 of the females and 3 of the males received the candidate vaccine *B. suis* VTRS2 expressing the mGnRH construct (~1 x 10^6 CFU VTRS2/mGnRHb administered IP), while the remaining animals received sterile saline. Six weeks post-inoculation all of the vaccinated mice received a booster (~1 x 10^5 CFU VTRS2/mGnRHb). The females were then introduced to male bedding beginning 3 nights prior to breeding and males were placed into individual cages a minimum of 6 hours before introduction of females. To be able to measure an effect of the vaccine on males, females, and the combined effect of both male and female vaccinates, harem breeding was performed in two rounds. First, all of the males were introduced to unvaccinated females (n=9 per male group, 3 females/male) and co-housed for 6 nights. Females were checked daily for the presence of a sperm plug, indicating they had been bred. After 6 nights, each female group was removed from the male and the male cage was changed for the second breeding. After a minimum of 6 hours, the vaccinated females were added to the male cages as described above. After 6 nights, vaccinated females were separated and males were euthanized via CO2 and cervical dislocation for necropsy.

At necropsy, testes and accessory sex organs were removed. Testis weights were recorded and each testis was placed in fixative containing 5% cold glutaraldehyde, 4.4% formaldehyde, 2.75% picric acid, and 0.05% sodium cacodylate buffer for H&E preparation at

VMCVM. Individual females were euthanized, weighed, and necropsied 17-18 days after detection of a sperm plug (pre-parturition). At necropsy, the reproductive tract was removed and the uterus was bisected to remove and count fetuses and evaluate fetal viability and number of fetal resorptions, as well as any other grossly observable abnormalities. The tract was then placed in 10% neutral buffered formalin for 5-7 days followed by 70% ethanol for H&E preparation at VMCVM.

Histopathology

All samples from female mice were evaluated for evidence of pathology by a board-certified veterinary pathologist at VMCVM. All samples from male mice were likewise assessed by the pathologist and were then scored based on the percentage of seminiferous tubule sections which contained degenerative vacuolation of the basement membrane.

Statistical analysis

All statistical analysis was performed using the JMP Pro 11 software (SAS). All continuous variables (testis weight, litter size) were analyzed using Student's T test and all proportion data (proportion pregnant) were analyzed using Chi-square and tested for probability of significance.

Results

General observation

Two of the three males in the double-treatment group breeding (H) had bite marks on their tails at necropsy. The uterus of five of the nine open vaccinated females had a bright red thickened appearance at necropsy. Additionally, the vaccinated females had higher abdominal fat relative to the unvaccinated group. These observations are most likely due to the older age at

necropsy of the vaccinated females. Unvaccinated male M2 failed to impregnate any of the six females he was housed with. There were no noticeable changes between groups on histopathology associated with the female reproductive samples. There was some evidence of degenerative changes in the male seminiferous tubule cross-sections; however, when degenerative vacuoles were quantified there was no difference between groups.

Testis weight

The mean weight of the paired testes of the vaccinated male group was 0.27g +/- 0.02 and the mean weight of the vaccinated male group was 0.22g +/- 0.01 (Table 4-2). This result was statistically significant via Student's T test (P=0.04); however, due to the small sample size the power of the test was low. There was no difference in body weight between the male groups.

Pregnancy rate

The mean pregnancy rate across the study was 61% and there was no significant difference between groups (Figure 4-1). The data were analyzed both with and without the infertile control male M2 and with the male groups compared with combined data from each breeding to remove influence of vaccination in the female on pregnancy rate for each male.

Litter size

The double-unvaccinated group (E) had the highest litter size (7.5 pups) and the vaccinated females bred to untreated males had the lowest (5.67 pups). Group E also had 0.75 more pups than group H (the double-vaccinated group) and the females-only vaccinated group (F) had one more pup than the double-vaccinated group; however, the data were not significant. Groups were also combined across both matings to compare the effect in males versus females without the influence of vaccination status in the opposite sex (Figure 4-2).

Fetal resorption

At necropsy, resorbed and non-viable fetuses were counted, in addition to pups. A fetus was considered non-viable if it was similar in size to a viable at-term fetus, but was pale and lacking a blood supply. A resorption was considered any fetal tissue smaller in size than a near-term fetus and lacking in both blood supply and normal physical appearance. Resorptions ranged from small discoid or amorphous uterine structures to incompletely formed, though recognizable, fetuses similar in size to the normal.

The double-vaccinated group (H) had the highest average number of resorptions (2.25 ± 0.92) especially compared to the unvaccinated female groups E (0.50 ± 0.29, P=0.11) and G (0.57 ± 0.30, P=0.075) (Figure 4-3). When all vaccinated males versus all unvaccinated females were compared, the results were similar though still not significant (P=0.052; Figure 4-4).

Discussion

Based on these results, vaccination with strain VTRS2/mGnRHb does not have a significant effect on fertility in mice. It has been previously shown that VTRS2/mGnRHb is able to elicit mGnRH IgG antibodies after single vaccination though the increase in antibody production against the delivered antigen does not appear to initiate any measurable effects on reproduction. However, the testis weight data merit further investigation in a more refined study. The biggest limitation of the pilot breeding trial was the study size. The number of animals used was mainly limited by the restraints of working under ABSL-3 conditions. While some differences between groups may have been biologically significant (testis weight, litter size, and resorptions in particular), the limitations of comparing small sample sizes precluded statistical significance. Another major factor in the results is the apparent infertility of one of the control

mice (M2). Mouse M2 did not impregnate any of the females he was exposed to, suggesting either infertility or lack of libido as the reason for this finding. A sperm plug was detected in all of the females he was exposed to, which makes infertility the most likely cause. Infertility is a common problem in inbred mice, including some lines within BALB/c (14-16), though a specific underlying cause was not determined in the individual mouse affected. To remove the influence of the infertile individual, the proportion pregnant was analyzed both with and without the inclusion of mouse M2; however, the sample size was too small for a significant result. Likewise, parameters specific to only the mice that got pregnant, such as litter size and number of resorptions per pregnancy, remove the influence of any mice that did not conceive. Along with testis weight, litter size was the parameter which displayed the greatest difference between vaccinated and unvaccinated groups. Though the data were not significant, the unvaccinated group had the highest litter size and the group with vaccinated females bred to unvaccinated males had the lowest. This trend was not reflected in the double-vaccinated group, or when vaccinated female group data was combined and compared with unvaccinated female groups across both matings (Figure 4-2).

To accommodate the limitations of working in ABSL-3 conditions and to reduce the total number of animals required for the study, breeding was done in two cycles using the same males. Pregnancy rates were not different based on timing of breeding (first versus second round of breeding). This validates the study design itself by demonstrating no difference based on either age of the females, or first versus second mating with the males. Any error attributable to first-time mating was likely avoided by leaving the males with the females for a full estrous cycle. As previously discussed, the main factor contributing to the lack of statistical significance is the relatively small sample size as very high numbers of individuals are required to detect

differences between proportions pregnant. Given the sample sizes that are reasonable to achieve, other parameters, such as litter size, carry more weight when examining the breeding study data, though they were insufficient to conclude that vaccination had an effect on fertility.

The testis weight between the treated and untreated groups was significant; however, due to the sample size limitation it is difficult to claim biological significance because there is too much type II error to confidently reject the null hypothesis that VTRS2/mGnRHb has no difference on testis weight in male mice. To further test this hypothesis, a subsequent study using only male mice was performed (See Chapter 5). This refinement study utilized a larger sample size, refined formulations of the VTRS2/mGnRHb vaccine, and was performed without the added variables associated with exposure to the female. Continuous variables such as testis weight, antibody titer, and testosterone level also allow for a more objective and confident statistical analysis of the data. This subsequent study was designed to more definitively test the hypothesis that vaccination with strain VTRS2/mGnRHb can confer an infertility defect in the mouse model. It was previously demonstrated that vaccination with VTRS2/mGnRHb led to an antigen-specific IgG response in female BALB/c mice (See Chapter 3). This is required in order for the body to mount an immune response against endogenous GnRH, thereby disrupting fertility. A likely cause of the apparent failure of the vaccine to cause a deficit in fertility in the breeding study is that despite a response being generated against the recombinant multimeric antigen, a subsequent response against endogenous hormone fails to develop. This hypothesis is further tested in the refinement study by ELISA measurement of GnRH antibodies in mouse plasma along with further assessment of reproductive parameters. While the results of the current study do not support the hypothesis that VTRS2/mGnRHb is able to confer infertility in

vaccinated mice, due to the difference observed in testis weight among the treated groups, further evaluation is indicated.

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Chapter 4 Tables and Figures

Table 4-1: 2x2 Breeding Study Design

2x2 Breedings	Vaccinated Males (Group N, n=3)	Unvaccinated Males (Group M, n=3)
Vaccinated Females (n= 9+9)	Group H_{1-3} n= 3 (3 females/male)	Group F_{1-3} n=3 (3 females/male)
Unvaccinated Females (n= 9+9)	Group G_{1-3} n=3 (3 females/male)	Group E_{1-3} n=3 (3 females/male)

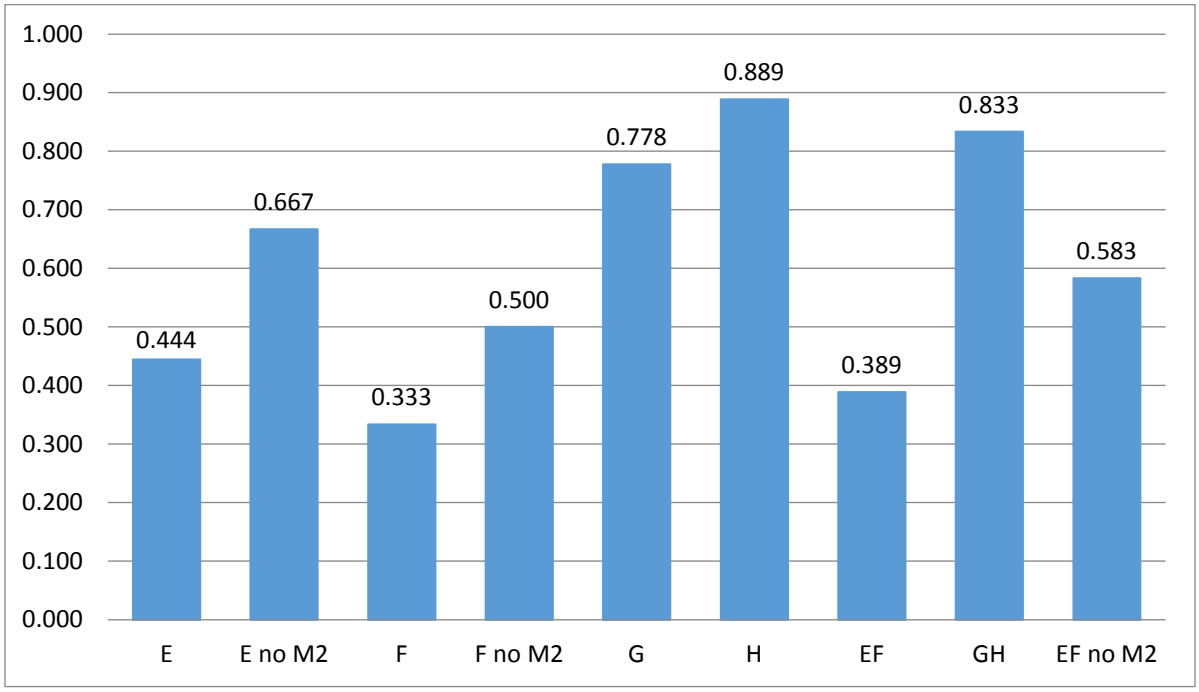
Two rounds of breeding were performed with each group of males, first they were bred to unvaccinated females G or E, then they were bred with vaccinated females H or F. 8-10 week old BALB/c mice were used.

Table 4-2: Mean testis weight

Unvaccinated males (M)	0.27g ± 0.02	
Vaccinated males (N)	0.22g ± 0.01	$P = 0.0423$

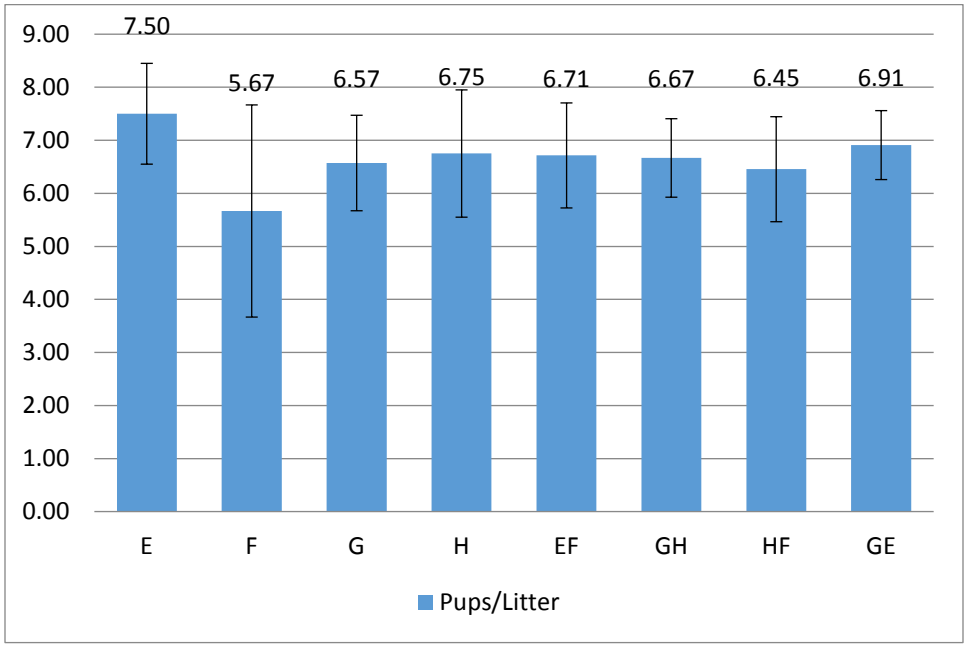
Mean testis weight; *P*-value generated via Student's T test

Figure 4-1: Proportion of females pregnant by group



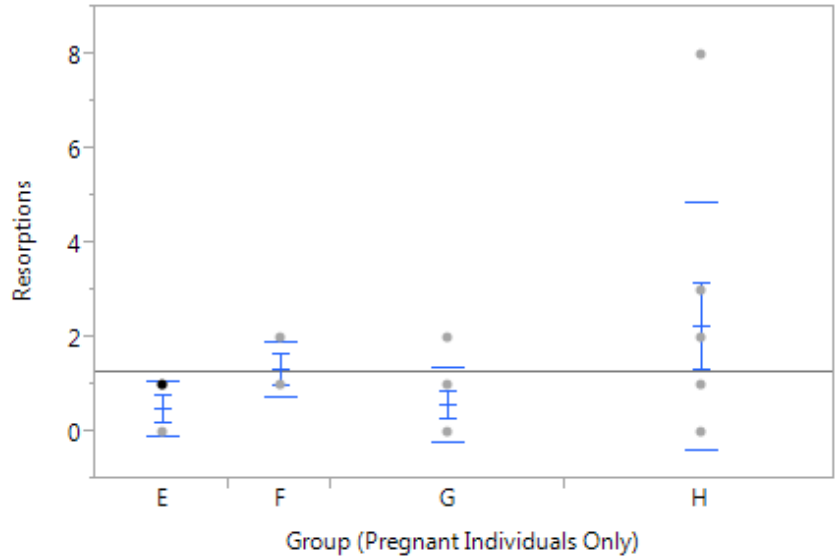
Data presented both with and without females bred to individual M2, which was apparently infertile; Groups- E (control; unvaccinated males and females), F (unvaccinated males, vaccinated females), G (vaccinated males, unvaccinated females), H (both males and females vaccinated), other groups are combined data to assess the effect in males across both matings (GH vs EF). There was no detectably significant difference between groups.

Figure 4-2: Litter size by breeding group



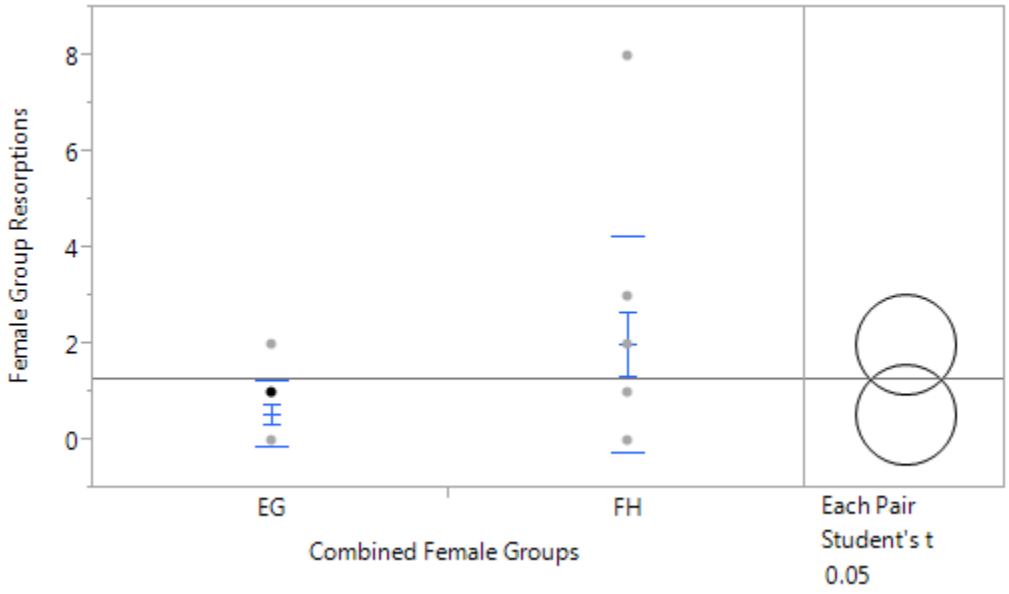
Litter size based on breeding group; Groups- E (unvaccinated males and females), F (unvaccinated males, vaccinated females), G (vaccinated males, unvaccinated females), H (both males and females vaccinated), other groups are combined data to assess the effect in all males (GH vs EF) and all females (HF, GE).

Figure 4-3: Fetal Resorption by breeding group



Number of resorbed or non-viable fetuses among pregnant females. Groups – E (unvaccinated females, unvaccinated males), F (vaccinated females, unvaccinated males), G (unvaccinated females, vaccinated males), and H (vaccinated females, vaccinated males).

Figure 4-4: Fetal resorption per litter among vaccinated versus unvaccinated female groups



Number of resorbed or non-viable fetuses per litter among Control (EG) versus Treated (FH) pregnant females (P = 0.052).

CHAPTER 5: EVALUATION OF LIVE-RECOMBINANT IMMUNOCONTRACEPTIVE VACCINES IN THE MOUSE MODEL AND THEIR POTENTIAL USE IN THE CONTROL OF FERAL SWINE

Abstract

Gonadotropin-releasing hormone (GnRH)-based immunocontraceptive vaccines have previously been shown to confer infertility in nuisance wildlife species such as feral swine (*Sus scrofa*). Feral swine populations are rapidly expanding in the United States and are a major cause of environmental, agricultural, and personal property damage where they have become established. Wild pigs are also the most widespread remaining reservoir of brucellosis in the U.S., and reintroduction of the disease into domestic herds could have lasting consequences on the beef and pork industries. Brucellosis is of particular concern because of its potential as a debilitating zoonosis. The *Brucella suis* vaccine strain VTRS2 has been shown to protect against virulent *B. suis* challenge in the mouse model and is capable of expressing recombinant antigen without the use of antimicrobial resistant markers. One such antigen is the multimeric recombinant GnRH construct mGnRHb. Vaccination with strain VTRS2/mGnRHb is able to elicit an IgG response against the recombinant antigen in BALB/c mice, but appears insufficient at compromising reproduction. In this study, six different formulations of VTRS2 carrying the mGnRHb antigen were tested in male BALB/c mice for their ability to elicit a response against endogenous GnRH and confer dysregulation of the endocrine reproductive pathway in the mouse model. A vaccine which protects against brucellosis and helps to reduce the fertility of the host could be a valuable tool to add to the resources available to wildlife management agencies for the control of nuisance feral swine and other pest species.

Introduction

Efforts to eradicate the disease brucellosis from the United States began as early as 1934 and it was not until the year 2000 that no positive herds were detected in cattle (1). Since that time the disease, caused by members of the Gram negative genus of bacteria *Brucella*, has been fully eradicated from domestic cattle and pigs; however, full eradication of the disease in the United States is impossible without controlling the spread of disease from wildlife reservoirs (2). Natural infections with *B. suis* and *B. abortus* in bison (*Bison bison*), elk (*Cervus elaphus*), and feral swine (*Sus scrofa*) continue to threaten domestic herds. Of these species, feral swine are the most widespread and have been implicated in *Brucella sp.* infection in both humans and domestic livestock (3, 4). Furthermore, feral swine are a damaging nuisance species whose population is estimated to have quadrupled since 2002 (5). The current methods of feral swine control are largely limited to hunting and trapping, which have proven insufficient at controlling the growth of the population and it is estimated that 85% of juvenile pigs need to be removed annually to prevent further population expansion (6).

Immunocontraception is one tool which has been proposed as a non-lethal aid to wildlife management agencies in controlling the population of wild pigs (7). Immunocontraceptives rely on the delivery of antigenic variations of endogenous components of the host reproductive system which elicit an autoimmune response against the molecule in the host (8). One of the most promising targets for this technique is gonadotropin releasing hormone (GnRH), a decapeptide released from the hypothalamus which acts on the pituitary as the highest level of regulation of reproduction in the body (9, 10). To date, the most successful of these vaccines have been subunit formulations which have major drawbacks such as being highly expensive to

produce and administer (11). Bacterial synthesis of antigenic peptide using recombinant DNA technology is one way in which cost can be reduced. This has been previously tested in feral swine using a recombinant multimeric GnRH-based vaccine termed the “Talwar recombinant”.

The multimeric GnRH (mGnRH) utilizes repeating GnRH decapeptide units interspersed with known T non B-cell epitopes from other well-known antigens such as tetanus toxoid (11-13). Despite being lower cost than conjugated peptide vaccines such as GnRH-keyhole limpet hemocyanin (GonaCon), mGnRH is still purified from bacterial culture and refolded for subunit delivery (11, 14). Live bacterial antigen delivery using recombinant technology has been shown to enhance protection of vaccines such as the widely used brucellosis vaccine RB51 (15). Previously, mGnRH has been shown to be adaptable for expression in the *B. suis* vaccine strain VTRS2 (this work). However, the resulting strain VTRS2/mGnRHb was unable to cause a decrease in pregnancy rate or litter size in a pilot breeding trial in the mouse model (see chapter 4). To further test the ability of VTRS2 as a delivery vehicle for immunocontraceptive antigen capable of compromising normal reproductive regulation, a study was designed for male mice using VTRS2/mGnRHb as well as 5 additional refinements of the original strain. The refinements were aimed at driving the host immune response from the Th1 cell-mediated immunity predominantly elicited by brucellosis vaccines to a predominantly Th2 humoral response as is required for immunocontraception. To determine the effect of vaccination on reproductive function, testosterone and anti-GnRH antibody levels were measured and mice were assessed for evidence of testicular degeneration via histopathological examination.

Materials and Methods

Vaccine strains

All experiments using *Brucella sp.* were performed in an approved biosafety level 3 (BSL-3) facility at VMCVM. *B. suis* VTRS2 strains containing empty expression vector pNS4 or the codon-optimized mGnRH containing plasmid pNS4*trcD*/mGnRHb (VTRS2/mGnRHb) were prepared as before (See Chapters 2 and 3). The drug resistance markerless expression plasmid pNS4 with constitutive hybrid *trcD* promoter (pNS4/*trcD*) was used for all constructs. The flgE signal sequence-containing plasmid pNS4*trcD*/flgE-mGnRHb was created using pNS4*trcD*/flgE (16) with mGnRHb inserted as described in Chapter 2. Plasmids pNS4*trcD*/flgE-mGnRHb-GFP and pNS4*trcD*/mGnRHb-GFP were created via insertion of a green fluorescence protein (gfp)-containing cassette downstream of mGnRH using SmaI and XbaI restriction sites. All plasmids were transformed into *B. suis* VTRS2 via electroporation and verified for expression via western blotting and Sanger sequencing (Virginia Bioinformatics Institute, now the Biocomplexity Institute of Virginia Tech). GFP-containing constructs were also verified for fluorescence under ultraviolet light.

Prior to inoculation, VTRS2/flgE-mGnRHb was also prepared with an equal dose of killed cells. This was performed via serial dilution of culture in sterile saline to 5 x 10^7 CFU/ml and splitting of the diluted culture. One milliliter of 5 x 10^7 CFU/ml was then boiled in sterile saline for 20 minutes, then lysate was then centrifuged at 12,000 x G in a microcentrifuge to pellet the lysed cellular material. The supernatant was decanted and the pellet was resuspended in 1 ml of 5 x 10^7 CFU of live culture resulting in equal parts killed and live organisms. An

additional formulation was prepared prior to inoculation by preparation of 5 x 10^7 CFU VTRS2/flgE-mGNRHb with 0.05% P85 polymer adjuvant (see Table 5-1 for the strains used).

Animal use

All animals used were male BALB/c mice (Harlan Laboratories) approximately 9 weeks of age at the start of the study (post-pubertal). Mice were housed in a CDC-approved Animal BSL-3 facility and cared for by the VMCVM Teaching and Research Animal Care and Support Staff (TRACSS). All experimental procedures were performed under an approved protocol by the Institutional Animal Care and Use Committee. Animals were euthanized via CO_2 asphyxiation followed by cervical dislocation. All blood collections were done via retro-orbital plexus venipuncture under isoflurane anesthesia to ensure collection of an adequate volume of plasma.

Study design

To determine the effect of different mGnRH-containing VTRS2 formulations on reproductive parameters in the mouse model, 40 male BALB/c mice were divided into eight groups (n=5) and the first seven groups were administered 5 x 10^6 CFU (IP) of one of the following vaccines: VTRS2/pNS4GroE, VTRS2/mGnRHb, VTRS2/mGnRHb-GFP, VTRS2/flgE-mGnRHb, VTRS2/flgE-mGnRHb-GFP, VTRS2/flgE-mGnRHb + equal dose killed organisms, VTRS2/flgE-mGnRHb + 0.05% P85. The eighth group received 50 µl GonaCon (provided by the USDA-APHIS NWRC) IM as a positive control (Table 5-1).

Blood was collected as described for anti-GnRH enzyme-linked immunosorbent assay (ELISA) on weeks 0 (pre-inoculation) and 3 (post-inoculation) and all mice were boosted on week 6 with 5 x 10^5 CFU IP. The mice receiving GonaCon were also boosted on week 6 with 50µl IM. Blood was collected post-booster (week 7) and at the termination of the study at 15

weeks post-initial vaccination for anti-GnRH ELISA and testosterone assays. After terminal blood collection, all mice were euthanized and necropsy performed.

At necropsy, testes and accessory sex organs were removed. Body weight and testis weights were recorded and each testis was placed in 10% neutral buffered formalin for 5-7 days followed by 70% ethanol for histopathological examinations of thin sections stained with H&E preparation at VMCVM.

Anti-GnRH ELISA

Ninety-six well microtiter plates were coated with 1μg GnRH-acetate in carbonate buffer. Wells were washed four times in TBS-Tween and blocked for 1 hour using 5% skim milk in TBS. Plasma samples from weeks 3, 7 and 15 were diluted 1:10 and 50 μl/well were added and incubated for 4 hours at room temperature in duplicate. After primary incubation, samples were washed four times with TBS-Tween and incubated with 1:5000 horseradish peroxidase (HRP)-conjugated anti-mouse IgG diluted in TBS. Wells were again washed as before and each plate was developed using TMB peroxidase substrate and H_2SO_4 stop solution. Plates were read at 450nm on an ELISA microplate reader (VersaMax). Mean absorbance and standard deviation were calculated and means were compared using Student's T-test (JMP Pro 11).

Testosterone Assay

Testosterone levels were assessed via acetylcholinesterase (AChE)-based competitive ELISA for the VTRS2 pNS4, VTRS2/mGnRHb, VTRS2/mGnRHb-GFP, VTRS2/flgE-mGNRHb + p85, and GonaCon groups using week 15 plasma samples and a total testosterone ELISA kit (Cayman Chemical). All samples were diluted 1:10 and 1:50 and run in duplicate. For each sample and each dilution, 50μl were added per well and incubated with an equal volume of AChE-testosterone tracer and anti-testosterone antiserum. After incubation for two hours at room

temperature, the plate was washed five times with commercial wash buffer (Cayman Chemical) and developed by adding 200µl Ellman's Reagent to each well. The plate was developed for 60 minutes in the dark at room temperature and read at 405nm on an ELISA microplate reader (VersaMax). Standards were transformed and fit via logarithmic regression. Higher absorbance values correlate with a lower concentration due to decreased inhibition of absorbance from lower concentrations of testosterone in the sample. Mean testosterone level was calculated from the logarithmic regression fit formula [x = e^((lny)+0.080541)/-0.1843701)] and both mean calculated testosterone level and mean absorbance were compared using Student's T-test.

Histopathology

Thin sections of the fixed testis samples were prepared for histopathology via hematoxylin and eosin stain at the VMCVM Veterinary Teaching Hospital. Each sample was blinded and fifty seminiferous tubule cross sections were randomly assessed. Each cross section was assessed for presence of degenerative vacuoles, absence of normal tubule architecture, absence of two or more stages of spermatogenesis, presence of syncytial cells, or presence of two or more of the above abnormalities. After completion, each sample was unblinded and means generated for each parameter for each group as well as for total abnormalities observed. Mean proportional abnormalities were compared via Student's T-test (JMP) and total abnormalities for each group were compared to controls using the 2-sample z-test for proportions (epitools.ausvet.com.au).

Results

Body weight and testis weight

No difference was observed between groups for body weight (data not shown). No difference was observed in paired testis weight between the VTRS2/pNS4 controls and the mGnRHb groups ($P>0.05$). The positive control GonaCon mice developed grossly observable testicular atrophy and significantly lower paired testis weight than all other groups ($P<0.01$) (Figure 5-1).

Anti-GnRH ELISA

Three weeks after initial inoculation both GonaCon positive control group mice and mice which received 5 x 10^6 VTRS2/flgE-mGnRHb + 0.05% P85 demonstrated a significantly higher anti-GnRH IgG response than the negative control mice receiving VTRS2/pNS4 without the mGnRH immunocontraceptive construct (Figure 5-2). No other groups had a significantly higher response than the negative control. One week after booster inoculation (7 weeks after initial inoculation) at the end of the study (week 15) only mice which received GonaCon demonstrated a significantly higher specific IgG response than the VTRS2/pNS4 group (Figures 5-3 and 5-4).

Testosterone level

Testosterone level was reflected in absorbance data by lack of inhibition of AChE-conjugated testosterone binding to testosterone anti-serum in the well. Mean absorbance was calculated and means compared via Student's T-test (Figure 5-5). Known standard concentrations of testosterone were also used to calculate total plasma testosterone and mean calculated testosterone levels were compared in the same manner (Figure 5-6). Only samples from mice which received GonaCon had significantly higher absorbance and significantly lower calculated testosterone levels than the VTRS2/pNS4 controls. Concentrations in GonaCon mice

also were significantly different than those receiving VTRS2/mGnRHb in both comparisons. Calculated testosterone from the mGnRHb-GFP and flgE-mGnRHb + P85 groups was lower than pNS4 controls however the means were not significantly different.

Histopathology

Syncytial cells, basement membrane vacuolation, absence of more than two stages of spermatogenesis, and absence of normal tissue architecture were assessed in randomly counted seminiferous tubule lumen sections for each individual in the study. None of the mice receiving any of the VTRS2/mGnRH constructs had significantly more abnormalities compared to the VTRS2/pNS4 empty plasmid controls for any of the abnormalities assessed. Testis cross sections from mice receiving GonaCon had significantly more degeneration, syncytial cells, and cross sections without evidence of active spermatogenesis (two or fewer stages of spermatogenesis present with no evidence of elongation of spermatids). When all abnormalities were considered together, only mice receiving GonaCon had a significantly higher proportion of abnormal tubule sections via both T-test and Z-test for comparing proportions ($P< 0.05$) (Figure 5-7).

Discussion and Conclusions

It has been previously shown that *B. suis* strain VTRS2 is able to confer protection in BALB/c mice against virulent *B. suis* challenge and that VTRS2 strains expressing multimeric GnRH are capable of eliciting a significant humoral response against the mGnRH antigen (see Chapter 3). However, the candidate immunocontraceptive vaccine failed to confer an apparent infertility defect in a subsequent breeding trial (see Chapter 4). In this study, the candidate strain VTRS2/mGnRH as well as 5 refined versions of the vaccine were tested solely in male mice to more fully determine their effect on reproduction. The advantage of using male mice is that their

endocrine regulation is less cyclical than that of the female and therefore measurement of the mean level of a single hormone is less dependent on the timing of cyclical endocrine events. Also, the effects of infertility agents are more directly observable based on their histopathological architecture and mean testosterone levels. Removing the variables associated with breeding, such as synchronization and cyclicity of the females, and inherent differences in fertility among individuals affecting more than one experimental group, was an important refinement over the breeding study to better focus on the direct effects attributable to vaccination. The refinement experiment was also improved via the addition of the infertility agent GonaCon as a positive control. This addition allowed for both validation of the variables measured as well as a direct comparison between the effects of the experimental vaccines versus a known and well-characterized infertility agent which has previously been used in feral swine (11, 17).

Though live brucellosis vaccines elicit humoral antibody production, protection is predominantly dependent upon their ability to mount a strong cell-mediated immune response (18, 19); however, vaccines against GnRH depend on a strong humoral immune response against endogenous GnRH to be effective (7, 20). The refinements made to the VTRS2/mGnRH construct were performed with the goal of stimulating an enhanced antibody response against endogenous hormone. All peptides were expressed behind the synthetic hybrid *trcD* promoter in plasmid pNS4*trcD*, which confers increased levels of constitutive expression over the native *groE* promoter in pNS4 (21). Green fluorescence protein (GFP) was cloned downstream of mGnRHb in strains VTRS2/mGnRHb-GFP and VTRS2/flgE-mGnRHb-GFP. Though GFP itself is poorly immunogenic, it is commonly used as a fusion antigen in both immunological studies of humoral immunity and candidate vaccine studies (22-24). In addition to acting as a reporter

for antigen expression, GFP promotes the humoral antibody response. This has been particularly demonstrated in antigen-fusion studies in which a GFP-antigen fusion produced a greater anti-GFP and anti-antigen response than either protein alone (23). In the testosterone assay, mice receiving VTRS2/flgE-mGnRHb-GFP experienced decreased total testosterone, which was not statistically different from the negative control. Furthermore, mice receiving constructs with GFP did not develop significant levels of anti-GnRH antibody. A possible reason for this is "antigen distraction" due to the relatively large size of the GFP molecule versus that of the GnRH decapeptide (22).

Three weeks after initial inoculation, mice receiving VTRS2/flgE-mGnRHb plus the polymeric adjuvant Pluronic P85 demonstrated a significant anti-GnRH response. P85 is a low-toxicity amphiphilic polymer which has been shown to enhance the response of antigen presenting cells to circulating and local antigens (25). In a previous experiment evaluating the role of P85 in enhancing the efficacy of a subunit brucellosis vaccine, it was found that the adjuvant increased the humoral IgG response without increasing the major cell-mediated immune effector interferon-γ (25). While the early peak IgG response against GnRH was significant in the P85 group in this study, this did not correlate with any significant effect on testis weight or histological architecture. Like the GFP group, the P85 group experienced a reduction in testosterone, however the data were not significant. The significant initial antibody response without subsequent anamnestic response against endogenous GnRH may be attributable to the dramatic effect of the adjuvant on antibody production causing a non-specific increase in IgG. In previous experiments using P85 adjuvant with *Brucella* vaccines, administration of P85 alone was sufficient to cause a significant reduction in challenge strain titer (25). This highlights

the marked effect that P85 has on short-term immunity though it remains unclear why a subsequent response against endogenous GnRH was not observed.

The third method employed in an attempt to increase the anti-GnRH IgG response was to increase the amount of extracellular antigen versus the cytoplasmic location of mGnRHb in live VTRS2/mGnRHb. To do this, mice were inoculated with either equal amounts of live and killed strain VTRS2/mGnRHb or with mGnRHb expressed behind the export signal sequence *flgE* (VTRS2/flgE-mGnRHb or VTRS2/flgE-mGnRHb-GFP). The *flgE* signal sequence belongs to the translocated Flagella E hook-protein of *B. abortus* and has been previously shown to cause secretion of fused heterologous antigen in *B. abortus* RB51 (16, 26). While the culture supernatant of VTRS2/flgE-mGnRHb-GFP was fluorescent, indicating secretion of the antigen, there was no effect of antigen secretion on GnRH antibody production or subsequent assessment of indicators of reproductive health. This is consistent with previous efforts in which secretion was achieved, but there was no increase in the antibody response or protective efficacy between secreted and cytoplasmic antigen (16). Likewise, addition of killed bacteria to live VTRS2/flgE-mGnRHb as a combined live-attenuated plus bacterin vaccine had no effect on GnRH antibody production despite the increased total antigenic load.

In conclusion, while *B. suis* VTRS2 expressing mGnRH antigen is protective against virulent *B. suis* challenge, the candidate immunocontraceptive has an effect initially on testosterone levels, but fails to confer any defect in normal reproductive function. The key point of breakdown appears to be the fact that despite being able to elicit an anti-mGnRH response against the exogenous multimeric antigen, the antibody levels were not sustained and this did not translate into a persistent response against endogenous GnRH. Efforts to elicit an anti-GnRH IgG response by strain VTRS2/mGnRHb and refined versions of the same were similarly ineffective.

The most likely reason for the lack of a strong effect on reproduction is insufficient antigen production by the bacterial vaccine. For a strong reproductive effect to be observed in trials using mGnRH as a subunit vaccine in previous studies, multiple large doses of peptide needed to be delivered with a strong adjuvant (11, 27, 28). A third inoculation or higher dose may help to mitigate this challenge; however, a 3-dose vaccine would be impractical for field application in feral swine and pathogenicity concerns arise with the use of a higher dose of live-attenuated *B. suis*. Another possible cause of the failure to elicit an effective response against endogenous hormone could be that the mGnRH antigen is too different from the endogenous hormone due to the repeats interrupted by other known epitopes. This seems less likely, however, as subunit studies using purified mGnRH and a potent adjuvant have proven effective at conferring infertility in pigs and prostatic atrophy in rats (11, 12). A third explanation could be that the live VTRS2 vaccine strain is eliciting a strong Th1, cell-mediated response and inhibiting the Th2 response. While there continues to be a need for additional population control methods in feral swine, these studies in the mouse model have conclusively demonstrated that the mGnRH antigen expressed in VTRS2 is insufficient for this purpose. Alternative carrier antigens such as *Brucella* lumazine synthase (29) or the FliC flagellin protein of *Salmonella enterica* ss: *enteritidis* (30, 31) conjugated to the GnRH decapeptide could be used to generate a more GnRH-specific response. Another alternative could be the use of *Brucella neotomae* as the platform species. *B. neotomae* is more attenuated than VTRS2 and can be made to carry the pNS4 family of plasmids (Waldrop, Jain, and Sriranganathan; Virginia Tech, unpublished research). The use of a further-attenuated species such as *B. neotomae* would allow for a higher inoculation dose and therefore a greater total initial amount of delivered antigen. A potential drawback of *B. neotomae* however is the potential for a decrease in protection against *B. suis*

challenge though this has yet to be demonstrated. Despite the lack of an infertility response with strain VTRS2 delivering immunocontraceptive antigen, there continues to be a lack of a widely approved vaccine with efficacy against *B. suis* infection in swine and strain VTRS2 may have value in the effort to control brucellosis in feral swine in the United States and in both feral and domestic animals worldwide.

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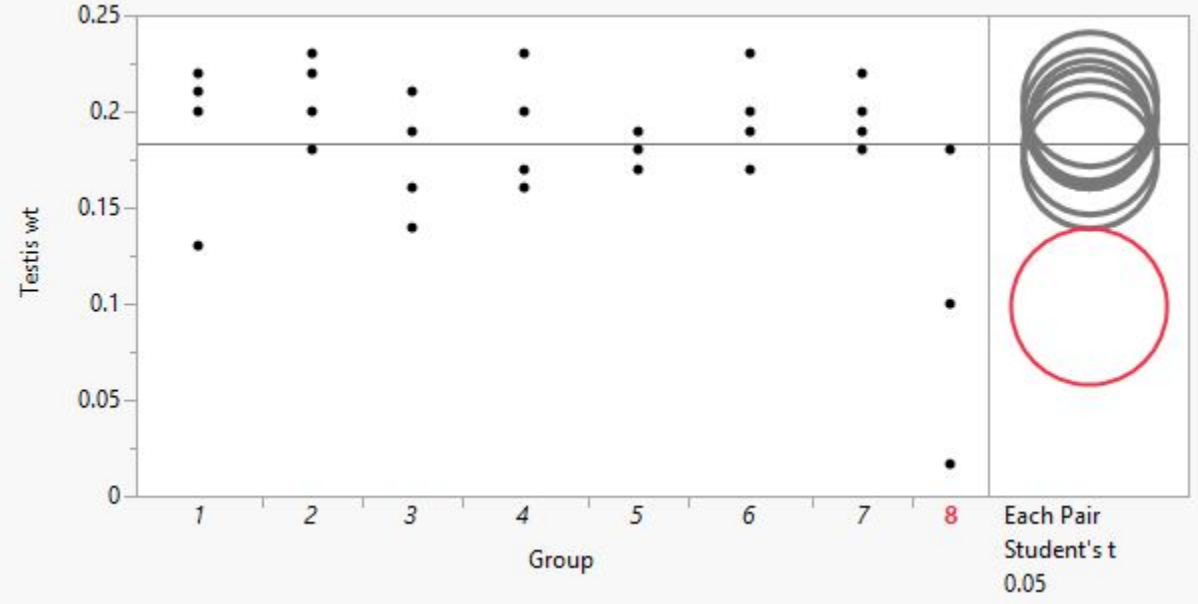
Chapter 5 Tables and Figures

Table 5-1: Groups used in the male contraceptive study

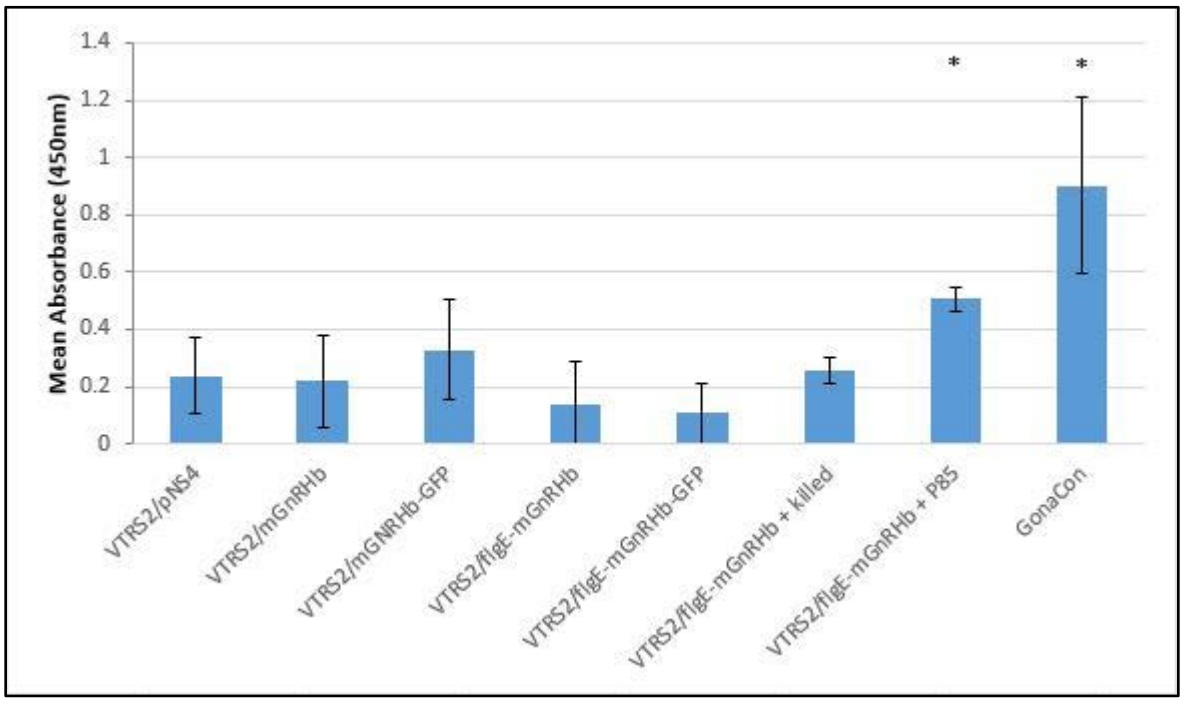
	MALE CONTRACEPTIVE STUDY 2015
	GROUPS: (n=5); Bacterial dose = 5 x 10^6 CFU IP
1	VTRS2/pNS4GroE
2	VTRS2/mGnRHb
3	VTRS2/mGnRHb-GFP
4	VTRS2/flgE-mGnRHb
5	VTRS2/flgE-mGnRHb-GFP
6	VTRS2/flgE-mGnRHb + killed preparation (equal dose)
7	VTRS2/flgE-mGnRHb + 0.05% P85
8	GonaCon positive control

Figure 5-1: Mean paired testis weight by group



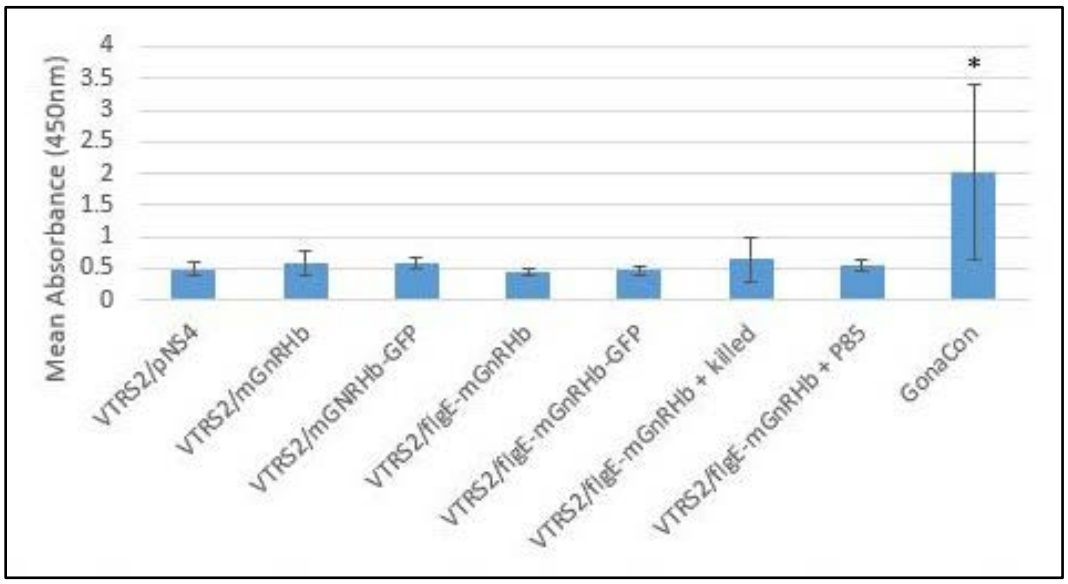
Paired testis weight (g) did not differ among groups VTRS2/pNS4 (1), VTRS2/mGnRHb (2), VTRS2/mGnRHb-GFP (3), VTRS2/flgE-mGnRHb (4), VTRS2/flgE-mGnRHb-GFP (5), VTRS2/flgE-mGnRHb + equal dose killed organisms (6), and VTRS2/flgE-mGnRHb + P85 (7). Mice, which received GonaCon (8) had significantly lower mean testis weight than all other groups ($P < 0.01$).

Figure 5-2: Anti-GnRH ELISA – Week 3



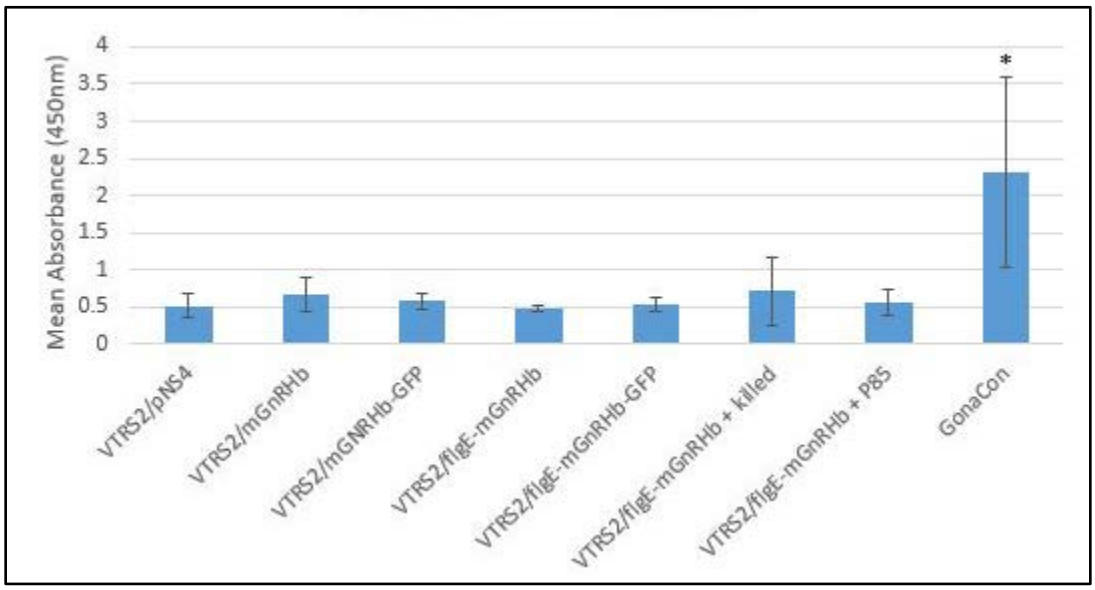
Anti-GnRH mouse IgG plasma antibody response 3 weeks post-inoculation – Mice receiving 5 x 10^6 CFU VTRS2/flgE-mGnRHb + P85 and mice receiving GonaCon demonstrated significantly higher anti-GnRH antibody levels than control mice receiving VTRS2/pNS4 alone ($P < 0.01$).

Figure 5-3: Anti-GnRH ELISA – Week 7



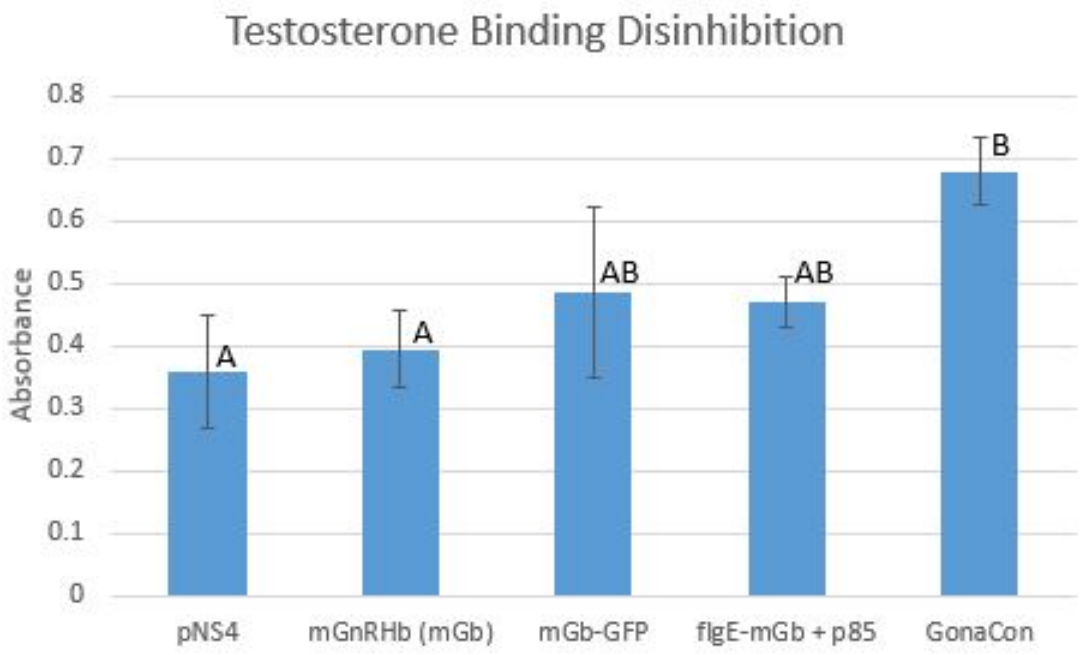
Anti-GnRH mouse IgG plasma antibody response 7 weeks post-inoculation and 1 week after booster inoculation – Only mice receiving two administrations of GonaCon demonstrated significantly higher anti-GnRH antibody levels than control mice receiving VTRS2/pNS4 alone ($P = 0.0407$).

Figure 5-4: Anti-GnRH ELISA – Week 15



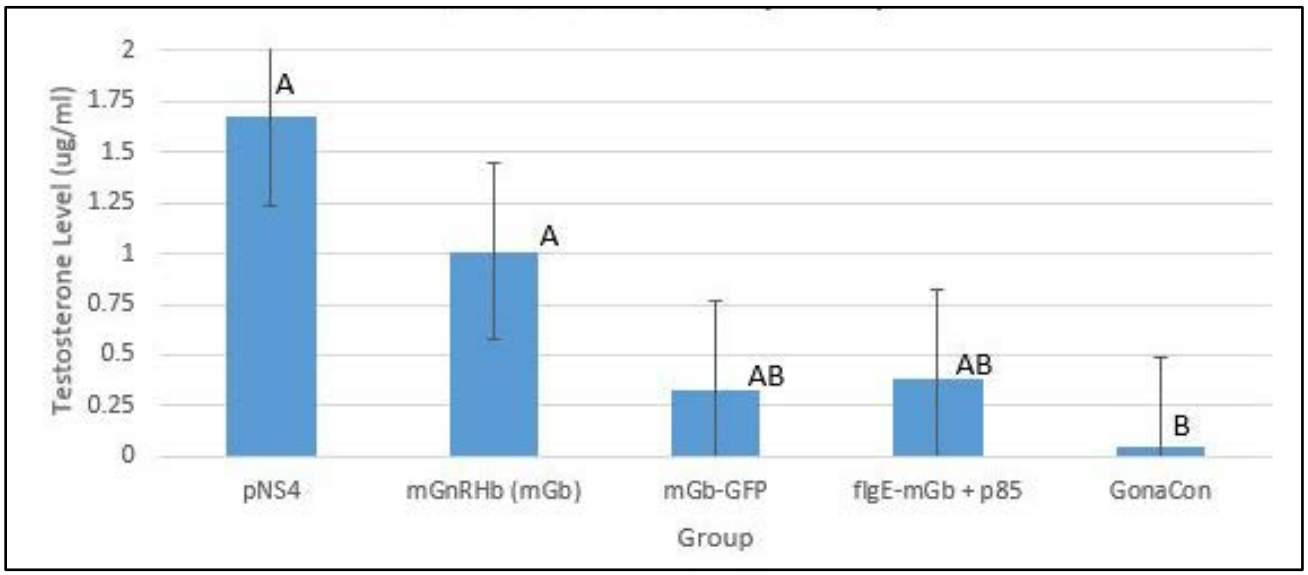
Anti-GnRH mouse IgG plasma antibody response 15 weeks post-inoculation – Only mice receiving GonaCon demonstrated significantly higher anti-GnRH antibody levels than control mice receiving VTRS2/pNS4 alone ($P = 0.0172$).

Figure 5-5: Testosterone Inhibition ELISA



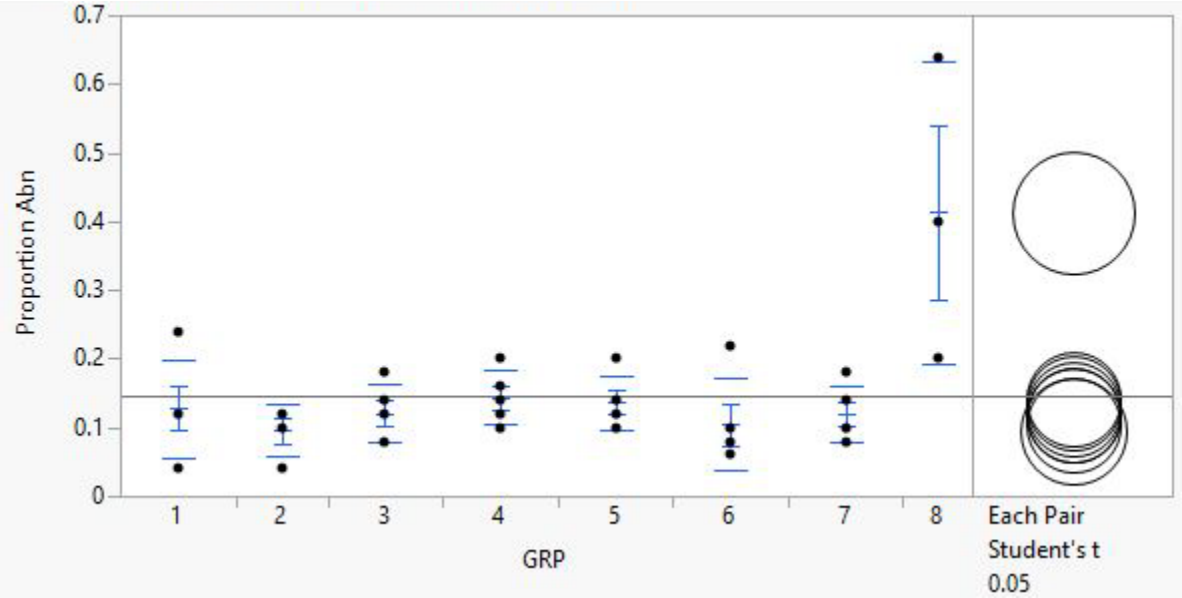
Higher absorbance reflects decreased inhibition of AChE-conjugated testosterone binding to anti-testosterone antiserum by plasma testosterone. Samples from mice, which received GonaCon had significantly less binding inhibition than the VTRS2/pNS4 control and VTRS2/mGnRHb groups. Common connecting letters signify lack of significance between groups (A is different from B but not AB). Binding was assessed via absorbance at 405nm, samples were diluted 1:10.

Figure 5-6: Calculated mean plasma testosterone level by group



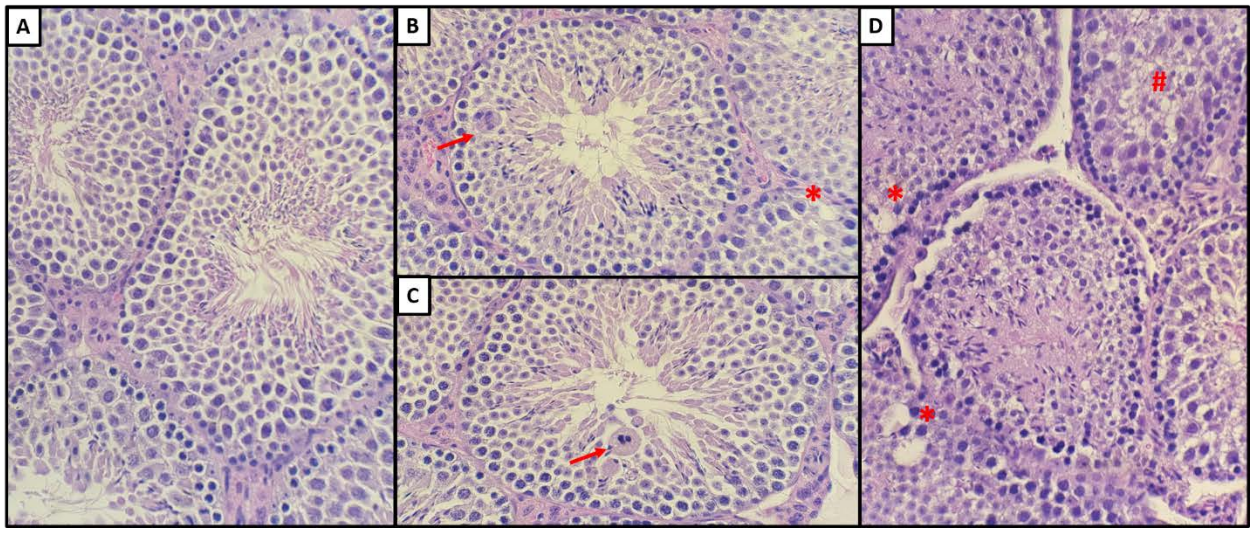
Testosterone level was calculated for each 1:10 diluted plasma sample based on the natural logarithmic fit of the standard curve using the formula:
[x = e^((lny)+0.080541)/-0.1843701)]
Mice receiving GonaCon had significantly lower total plasma testosterone levels than mice receiving VTRS2/pNS4 or VTRS2/mGnRHb. Mice receiving VTRS2/mGnRHb-GFP or VTRS2/flgE-mGnRHb + P85 had lower circulating levels however the results were not significant. Like-Connecting letters signify lack of a significant difference (A is significantly different from B but not AB).

Figure 5-7: Proportion of abnormal seminiferous tubule cross-sections



Comparison of proportion of abnormal seminiferous tubule cross sections in H&E prepared testis samples from mice receiving two inoculations of VTRS2/pNS4 (1), VTRS2/mGnRHb (2), VTRS2/mGnRHb-GFP (3), VTRS2/flgE-mGnRHb (4), VTRS2/flgE-mGnRHb-GFP (5), VTRS2/flgE-mGnRHb + equal dose killed organisms (6), and VTRS2/flgE-mGnRHb + P85 (7) or the subunit immunocontraceptive GonaCon (8). Only mice receiving GonaCon had a significantly higher total or proportion of abnormal tubule sections via both Student's T-test ($P < 0.0001$) and Z-test for comparing proportions ($P < 0.0001$). Data analysis was performed using JMP Pro Software (SAS).

Figure 5-8: Representative cross sections of normal and degenerate testis seminiferous tubules



Four representative histopathology cross-sections (40x objective) of mouse testis parenchyma. A – Normal architecture from a negative control mouse, note the progressive, organized seminiferous tubule architecture resulting in normal spermatocyte development; B – Representative images from two experimental group mice (VTRS2-mGnRHb) with subtle degenerative changes including multinucleate giant cells (Arrow), and basement membrane vacuolation (*). In image B, note the continued orderly progression of spermatogenesis as evidenced by multiple stages of development including elongated spermatids. While sections from experimental group mice have been used in this figure, subtle degenerative changes like those shown are also present in the tissue of normal mice at a normal low incidence; D – An image from the positive control (GonaCon) mouse in which multiple degenerative changes are evident including extensive basement vacuolation (*), obliteration of normal tissue architecture, and lack of progression of spermatogenesis (#).

GENERAL CONCLUSIONS

Feral swine continue to be a costly nuisance species across the United States and elsewhere around the world and it is widely agreed that additional control measures are needed to prevent further increase in their population. Furthermore, feral swine pose the threat of reintroduction of eradicated zoonotic diseases of livestock such as brucellosis. As the most widespread remaining sylvatic reservoir in the U.S., feral swine are of particular concern in brucellosis control efforts. There is no approved vaccine for use in pigs and previous efforts to achieve protection with existing approved vaccines used in cattle such as RB51 have been largely unsuccessful. Recently, steps were taken by Olsen et al. (2013) towards a *Brucella suis*-based rough vaccine for use in swine. The resulting strain, *B. suis* 353-1, is an attenuated naturally occurring rough stain which was isolated by USDA-NADC researchers from an infected feral pig in South Carolina. While 353-1 appears to protect against *B. suis* challenge and is phenotypically rough, it is a poorly characterized strain whose exact attenuating mutations are yet to be known. The advantage of synthetic auxotrophic deletion strains such as *B. suis* VTRS2 is that the mutations are well defined which may allow for a better understanding of the mechanisms of protection and attenuation, in addition to environmental tracking of the strains via molecular techniques such as PCR and RFLP analysis. The deletion mutation of gene *wboA* is one which has been especially well characterized previously and is known to attenuate *in vivo*. In a pilot study alongside *B. suis* 353-1 (courtesy of Dr. SC Olsen, USDA-NADC), strain VTRS2 appears to reduce challenge burden of virulent *B. suis*, though not as completely as 353-1 (See Addendum 1). The results from this study are encouraging for the prospect of a new tool against brucellosis in swine as well as for the platform strain VTRS2 as a delivery vector for heterologous antigen such as the mGnRH immunocontraceptive.

While the multimeric gonadotropin-releasing hormone (mGnRH) construct has been demonstrated to confer infertility in swine as a subunit-delivery vaccine, our studies were able to only partially duplicate this effect in the mouse model using live bacterial delivery in strain VTRS2-mGNRHb. Immunocontraceptive antigen delivery using live-attenuated *B. suis* vaccine would confer the benefits of reduced cost over current alternatives and additional protection against *Brucella* infection. Strain VTRS2-mGnRHb was able to elicit an anti-mGnRH IgG response in mice, however, this response did not translate to the auto-antibody response against endogenous GnRH, which is the hallmark of immunocontraception. The explanation for this is likely the fact that total antigen delivery is limited in live bacterial vaccines by the residual inherent virulence of the attenuated strain as well as the possibility of limited ability of the bacteria to produce heterologous antigen despite use of the strong constitutive promoter *trcD*. To elicit an auto-antibody response using subunit delivery in swine, as much as a gram of purified antigen is delivered alongside a potent adjuvant which is slow to clear from the host, allowing for prolonged enhancement of antibody production. This is expensive in commercial swine production and impractical in a wildlife vaccination program due to high cost of subunit vaccine, the requirement for parenteral delivery, and the unacceptable residues potentially associated with oil-based and other adjuvants. While live vaccine strains such as VTRS2 must persist in the host for some period of time to be effective, this time period is well-defined and limited by design (less than six weeks in the case of VTRS2) to ensure adequate attenuation. Adjuvants in subunit delivery vary greatly in their associated withdrawal period recommendations and may require long periods post-inoculation before the meat of injected animals is considered safe for human consumption. In a wildlife situation, particularly for feral swine, which often have very long or unlimited hunting seasons, this is a factor which must be considered. The easily-definable

withdrawal period of live vaccine via controlled post-mortem studies is an added advantage of VTRS2. For example, the clearance time demonstrated in the mouse model in this research could easily be established in the swine model using similar methodology, and vaccination at an appropriate time before the hunting season could be implemented.

In conclusion, strain VTRS2 has promise for further testing as a brucellosis vaccine in swine. Furthermore, the studies in this dissertation demonstrate that the strain can be used as a platform for heterologous antigen expression including novel immunocontraceptive peptides. While expression of the immunocontraceptive antigen mGnRH was able to induce only a partial auto-immune response, it did elicit a significant anti-mGnRH IgG response against the delivered antigen. Due to the inability of the VTRS2-mGnRH strain to confer fertility defects in the mouse model, alternative approaches to platform-delivered immunocontraception should be pursued. Expression of antigen using a more attenuated platform strain such as *Brucella neotomae ΔwboA ΔleuB* may allow for a higher dose of bacteria to be used and therefore also a higher antigen burden. Such an effort is being pursued in our laboratory currently with some encouraging results. Despite the limitations of subunit vaccines, purified GnRH antigen preparations have been previously shown to be effective in conferring infertility via immunocontraception. Based on the findings of these studies, delivery of brucellosis vaccine in conjunction with subunit immunocontraceptive is an alternative which merits consideration to aid in the continued effort to combat both the spread of disease and rapid population growth of feral swine. These and other alternatives should be explored in the continued search for an improved swine brucellosis vaccine and for additional methods for controlling the ever-expanding population of feral swine.

ADDENDUM ONE: PROTECTION AGAINST VIRULENT B. SUIS CHALLENGE USING B. SUIS STRAIN VTRS2 IN FERAL SWINE

Introduction:

This research is the result of generous inclusion of strain VTRS2 into a follow-up study by WC Stoffregen, CS Johnson, and SC Olsen (USDA-ARS) assessing the effects of a naturally acquired rough mutant (*B. suis* 353-1) in swine. The original study was published in 2013 by Stoffregen et al. (1) assessing the safety and immunogenicity of strain 353-1. The follow-up challenge study in both domestic and feral pigs using virulent *B. suis* strain 3B was subsequently performed using 353-1 either orally or parenterally delivered or parenteral VTRS2. A manuscript from that study was prepared without reporting on the results of the strain VTRS2 group and the findings were presented at the Conference of Research Workers in Animal Diseases (CRWAD) in 2013 (2). Inclusion of a VTRS2 group into the study design allows assessment of the strain as a potential vaccine against brucellosis in feral swine. The specific VTRS2 strain used was VTRS2 pNS4-GroE-mGnRH which carries the original Talwar mGnRH construct on the expression vector pNS4 as described by Givens (2010) (3). This construct was never demonstrated to be capable of expression of the mGnRH antigen in *B. suis* and was replaced with codon-optimized mGnRHb for the studies in the mouse model described elsewhere in this dissertation. Thus, the focus of this addendum is on the ability of VTRS2 to protect against virulent *B. suis* challenge in feral swine. The data from the VTRS2 group included herein is previously unpublished.

References and Attributions:

1. **Stoffregen WC, S. Johnson C, Olsen SC.** 2013. Immunogenicity and safety of a natural rough mutant of *Brucella suis* as a vaccine for swine. Research in Veterinary Science **95:**451-458.

2. **Olsen S, Nol, P., Rhyan, J.** 2013. Abstract: Immunogenicity and Efficacy of Oral or Parenteral Delivery of *Brucella suis* strain 353-1 to Domestic and Feral Swine, abstr Conference of Research Workers in Animal Diseases, Chicago, IL, 12/08/2013.
3. **Givens AR.** 2010. *Brucella abortus* RB51 Vaccine Over-expressing GnRH as an Immunocontracpetive for Feral Swine. M.Sc. Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA.

Materials and Methods:

All experiments were performed at the USDA-ARS National Animal Disease Center (Ames, IA) in an approved large animal biosafety level 3 (BSL3) facility. Domestic commercial-cross barrows (8-10 weeks of age, n=25) and similarly aged captive-born feral swine (mixed gender, n=25) were used. Feral and domestic animals were separately assigned to groups which received either no inoculation, approximately $2x10^{10}$ CFU *B. suis* 353-1 IM, approximately $2x10^{10}$ CFU 353-1 PO, or approximately$2x10^{10}$ CFU *B. suis* VTRS2 pNS4-GroE-mGnRH (VTRS2) IM.

Eighteen weeks following inoculation, the 353-1 groups were challenged with $5x10^{7}$ CFU virulent *B. suis* strain 3B. The VTRS2 groups were challenged similarly 8 weeks after vaccination. The feral and domestic groups were euthanized at 4 and 5 weeks post-challenge, respectively. At necropsy, aseptic collection of tissues was performed for processing and serial dilutions made for plating. The following tissues were sampled: lung, liver, spleen, kidney (major reticuloendothelial organs, RES), bronchial, hepatic, inguinal, internal iliac, mandibular, mesenteric, parotid, popliteal, prescapular, and retropharyngeal lymph nodes, and uterus, epididymis, testes, and bulbourethral glands (reproductive organs). The Retropharyngeal, mandibular, and parotid lymph nodes are collectively referred to as the cervical lymph nodes and are the major draining lymph nodes (LN) of the challenge inoculation site.

Approximately 1 gram of each harvested tissue was individually ground in 2 ml of sterile PBS and serially diluted to count CFU per gram of collected tissue following plating on TSA. Colonies were counted and tissues were grouped as cervical LN, distant LN, reproductive organs, and major RES organs for analysis. Groups were analyzed for incidence of infection by organ group, LOG CFU total per organ group, LOG CFU total per individual, and total mean reduction in LOG CFU versus the negative controls. Means were compared using general linear modelling and Dunnett's test (JMP Pro, SAS).

Additionally, peripheral blood was collected at 0, 4, and 8 weeks post-vaccination and at 2 weeks post-challenge to assess the humoral response of each group to the vaccine strain via antibody-capture ELISA. For ELISA, approximately $1x10^{10}$ CFU formalin killed 353-1 was used as antigen. Following coating of the plates with antigen, plates were washed and incubated with serum diluted 1:100 in quadruplicate. Detection of antibody was then performed using HRP-conjugated rabbit anti-swine IgG (1:500). Following development, response was measured as optical density (OD) and means were compared.

Results and Discussion:

None of the domestic pig groups showed evidence of challenge-strain colonization in either the major reticuloendothelial system organs or reproductive tissues. Challenge strain was isolated from cervical LN in 6 of the 7 control domestic pigs and from distant LN in 3 of 7 controls versus 5/7 and 4/7 LN in the domestic VTRS2 group, respectively (Table A1). The challenge strain was more disseminated in the feral pig groups, with isolation of strain 3B occurring in major RES organs of 9 of the 9 control pigs. The challenge strain was isolated from the major RES organs of 1 of 5 VTRS2-vaccinated feral pigs. Similarly, 8 of 9 control pigs had

colonization in their reproductive tissues versus 0 of 5 pigs in each of the vaccinated groups (Table A2).

Upon analysis of the mean total harvested CFU from the cervical lymphatic tissues, a 0.55 LOG CFU reduction was calculated versus unvaccinated controls in VTRS2 vaccinated domestic pigs though the data were not significant. Only the parenteral 353-1 vaccinated group displayed a significant difference in cervical tissue colonization following challenge (1.6 LOG reduction) (Figure A1). In the feral group, a 1.08 LOG reduction was calculated (Figure A2) using the mean total harvested CFU for all tissues. Greater than 1 LOG reduction is generally considered biologically significant protection however the means between the VTRS2 and control groups were not significant ($P = 0.11$). One individual in the VTRS2 group had particularly high titers which led to the lack of significance between the means. Reduction was greater in the parenteral (3.32 LOG) and oral (2.15 LOG) 353-1 vaccinated groups though there was only a significant difference in reduction between the parenteral 353-1 and VTRS2 vaccinated groups, not between pigs which received parenteral VTRS2 and those which received oral 353-1.

In the domestic pigs, a significantly higher level of humoral response to killed 353-1 antigen was observed via ELISA in the VTRS2 vaccinated pigs versus unvaccinated controls at all time points ($P < 0.05$). Additionally, the response was significantly greater in the VTRS2 group than either of the 353-1 groups 4 weeks after vaccination and was greater than the oral 353-1 group at all time-points (Figure A3). Interestingly, a higher baseline level of response was present prior to vaccination in the VTRS2 group. While the result is significant, the subsequently increased response at future time points should still be considered biologically significant.

In the feral pig groups, response to strain VTRS2 vaccination versus controls was measured at 8 weeks post-vaccination and two weeks post-challenge. The response was significantly greater in the vaccinated group at both time-points. No difference between the 353-1 and VTRS2 groups was noted in the feral pig groups at any time point (Figure A4).

Conclusions:

Strain VTRS2 was able to elicit a significant humoral immune response against *Brucella suis* in vaccinated pigs, however only marginal protection against virulent challenge was achieved based on collected culture data. Strain VTRS2 performed similarly to the naturally-derived rough strain 353-1 in reducing the incidence of infection of different tissue groups in feral pigs, though the titers of virulent *B. suis* in those animals with infected tissues were significantly lower in pigs parenterally vaccinated with strain 353-1 than with strain VTRS2. Both strains were similarly highly effective in preventing colonization of reproductive tissues, which may have an effect on reducing shedding in field conditions.

Strain 353-1, when parenterally delivered, was completely effective in reducing colonization by the challenge strain *B. suis* 1330. In the other groups, the titers in the cervical regional lymph nodes which drain the challenge inoculation site were higher than in other tissues. In the control group however, virulent challenge strain organisms were broadly disseminated to the reproductive organs and distant lymph nodes as well as the major RES organs lung, liver, spleen, and kidney. It is in these organs where strain VTRS2 showed the best protection. It is possible that VTRS2 is sufficient at causing enough protection to prevent broad dissemination in the host but not enough to elicit clearance of the challenge strain within two weeks. It is also possible that the decreased interval between vaccination and challenge in the strain VTRS2 versus strain 353-1 groups prevented either complete clearance of the vaccine

strain or adequate generation of a maximum immune response. Longer vaccination-challenge intervals are not typically associated with improved protection, so it is likely that a combination of factors related to the clearance kinetics of strain VTRS2 are involved in the observed differences.

Another factor of importance is the VTRS2 strain used in the study carries the plasmid pNS4-groE-mGnRH. While this plasmid was not shown in earlier studies (unpublished research) to express biologically active mGnRH antigen in *B. suis*, it is possible that a truncated peptide was still produced by the moderately strong constitutive groE promoter. Our work has shown that the original mGnRH Talwar construct carries several codons for which *Brucellae* lack sufficient tRNAs for translation. Stress from the organism producing truncated protein could be a factor in the survival of strain VTRS2 and decreased protection versus the naturally occurring strain 353-1. Despite this potential stressor, VTRS2 was still able to elicit some protection in feral pigs. Furthermore, the VTRS2 laboratory strain is well characterized *in vitro* and capable of expression of homologous or heterologous antigens which could increase protection without the addition of antibiotic-resistance markers. The insertion of a loxP scar into the mutation sites (see Chapter 2) also provides a signature which could be used for differentiation of vaccine strain in a field setting in which other rough *Brucella* such as strain 353-1 have been identified as part of naturally occurring infections.

These findings warrant further exploration of both strain VTRS2 (with or without homologous antigen expression plasmid) and strain 353-1 in swine. The ability of strain 353-1 to provide protection via oral vaccination is especially promising for field application. Assessment of the clearance kinetics of strain VTRS2 in feral swine may allow for a better side-by-side comparison of the two strains in subsequent challenge studies. There is no current approved

vaccine against brucellosis in pigs and feral swine are the most widespread remaining reservoir of the disease in the United States. The potential benefits of each strain merit consideration for further development as candidate vaccines for use against brucellosis in feral swine, as full eradication of the disease in the US cannot be achieved without eradication from wildlife reservoirs. Strain VTRS2 is a strong candidate for over-expression of homologous as well as heterologous antigens in these efforts.

Addendum 1 Tables and Figures

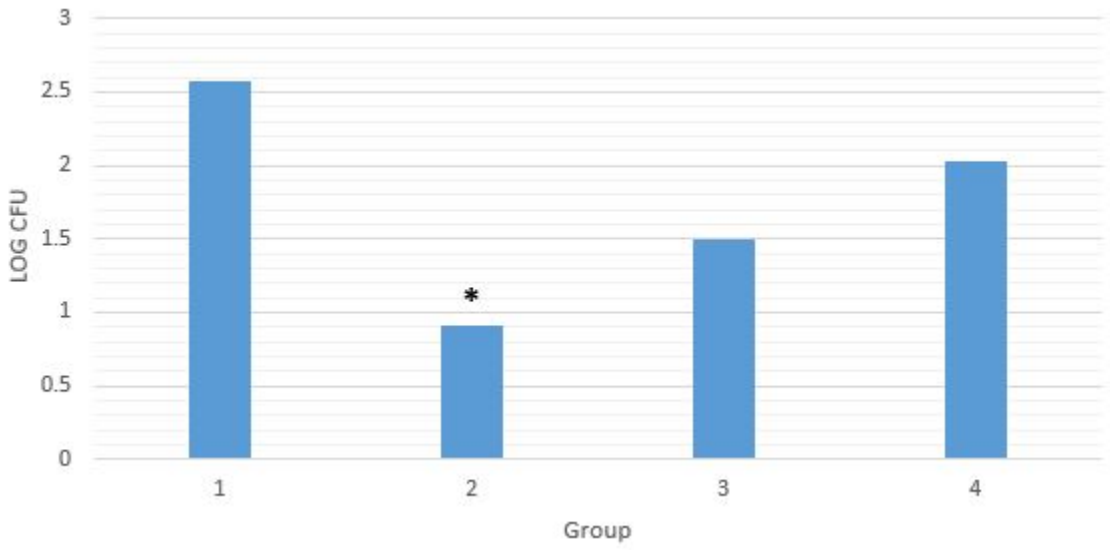
Table A1 – Incidence of Infection in the tissues of domestic pigs challenged with *Brucella suis* strain 3B

	Control	353-1 (parenteral)	353-1 (oral)	VTRS2 (parenteral)
Major RES Organs	0/7	0/6	0/5	0/7
Reproductive	0/7	0/6	0/5	0/7
Cervical Lymph Nodes	6/7	3/6	3/5	5/7
Other Lymph Nodes	3/7	1/6	0/5	4/7

Table A2 – Incidence of infection in the tissues of feral pigs challenged with *B. suis* strain 3B

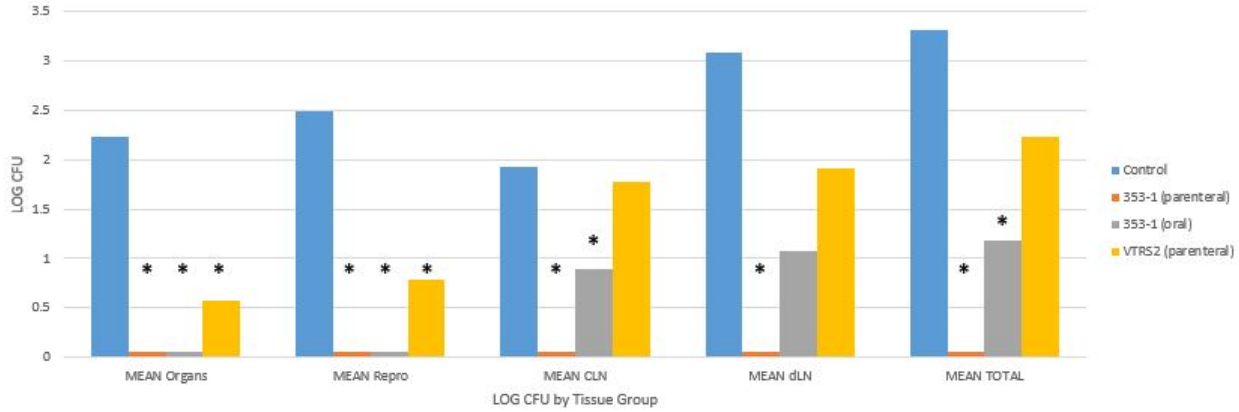
	Control	353-1 (parenteral)	353-1 (oral)	VTRS2 (parenteral)
Major RES Organs	9/9	0/5	0/5	1/5
Reproductive	8/9	0/5	0/5	0/5
Cervical Lymph Nodes	7/9	0/5	2/5	4/5
Other Lymph Nodes	9/9	0/5	2/5	3/5

Figure A1 – LOG CFU harvested from the cervical lymphatic tissues of domestic pigs challenged with *B. suis* strain 3B



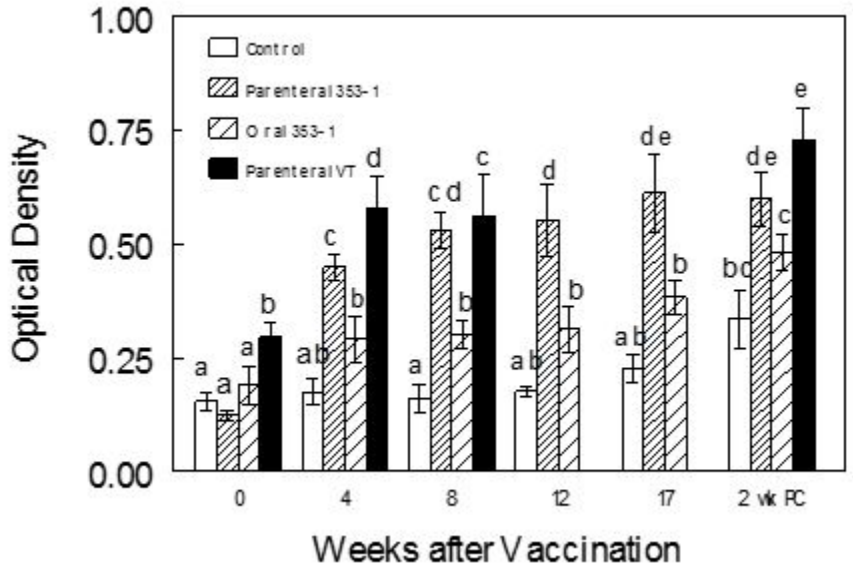
Mean LOG CFU of virulent *Brucella suis* from total cervical lymph node samples in vaccinated domestic pigs 2-weeks post-challenge. 1 – Negative control, 2 – *B. suis* 353-1 (parenteral), 3 – *B. suis* 353-1 (oral), 4 – *B. suis* VTRS2. * = $P < 0.05$

Figure A2 – LOG CFU harvested from the tissues of feral pigs challenged with *B. suis* strain 3B



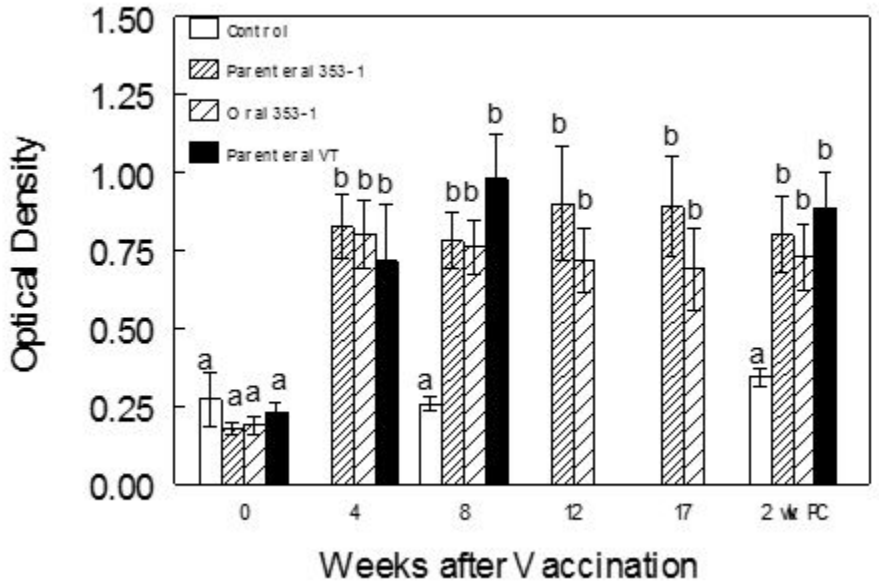
Mean LOG CFU of virulent *B. suis* 3B isolated from tissues of vaccinated feral pigs. Mean Organs – liver, lung, spleen and kidney; Mean Repro – uterus, testes, epididymis, inguinal lymph node, and internal iliac lymph node; Mean CLN – lymph nodes draining the challenge site at the bulbar conjunctiva (submandibular, retropharyngeal, and parotid); Mean dLN – lymph nodes distant to the challenge inoculation site (bronchial, hepatic, iliac, mesenteric, popliteal, inguinal, and pre-scapular); Mean Total – mean of the sum CFU of all collected tissues. * = $P < 0.05$ for means compared to the unvaccinated negative controls

Figure A3 – Humoral response to incubation with killed *B. suis* 353-1 in experimentally vaccinated domestic pigs (figure courtesy of Dr. Steven Olsen, USDA-ARS)



Humoral response of vaccinated and unvaccinated domestic pigs to killed *B. suis* 353-1 antigen. Diluted serum (1:100) from pigs exposed to VTRS2 displayed a significantly higher anti-*Brucella* IgG response than controls at all measured time points, including 2 weeks after challenge. Following challenge, VTRS2 delivered intramuscularly elicited a greater IgG response than oral *B. suis* 353-1. Pigs receiving VTRS2 were challenged 8 weeks after vaccination and therefore do not have time points at the 12 and 17 week sample dates. All samples were diluted in quadruplicate and means with differing connecting letters are statistically different ($P < 0.05$)

Figure A4 – Humoral response to incubation with killed *B. suis* 353-1 in experimentally vaccinated feral pigs (figure courtesy of Dr. Steven Olsen, USDA-ARS)



Humoral response of vaccinated and unvaccinated feral pigs to killed *B. suis* 353-1 antigen. Diluted serum (1:100) from pigs exposed to VTRS2 displayed a significantly higher anti-*Brucella* IgG response than controls at all measured time points, including 2 weeks after challenge. Pigs receiving VTRS2 were challenged 8 weeks after vaccination and therefore do not have time points at the 12 and 17 week sample dates. All samples were diluted in quadruplicate and means with differing connecting letters are statistically different ($P < 0.05$)