Development and testing of recombinant *B. abortus* RB51 vaccine strains carrying *M. tuberculosis* protective antigens

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Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis,* is one of the most prevalent infectious diseases inflicting humankind. The World Health Organization estimates that one third of the world's population, approximately 2.2 billion people, is infected with TB with a mortality of 1.7 million people annually. Currently, the WHO estimates that each year more than 9 million people develop TB.

Bacille Calmette-Guérin (BCG), an attenuated strain of *M. bovis,* is the only licensed TB vaccine in the world. Clinical studies have shown childhood vaccination with BCG to be protective against disseminating and meningeal forms of TB. However, the efficacy of BCG against pulmonary TB in adults has been variable and inconsistent (0-80%).

The objective of this study is to develop and test the efficacy of the *B. abortus* vaccine strain RB51 as a platform for expression of *M. tuberculosis* antigens (Ag85B, ESAT6 and Rv2660c) and induction of a protective immune response against *M. tuberculosis* and *B. abortus* challenge in mice.

Here we report the construction of two recombinant strains of *B. abortus* vaccine strain RB51 capable of expressing mycobacterial antigens Ag85B, ESAT6 and Rv2660c. Our studies show that expression of mycobacterial antigens in strain RB51 lead to induction of antigen-specific immune responses characterized by secretion of IgG2a antibodies as well as of IFN-γ and TNF-α. Mice immunized with a combination of two strains of RB51 in equal numbers, one carrying Rv2660c-ESAT6 and another carrying Ag85B, led to a 0.90 log reduction in CFU burden with significance nearly reaching borderline ($p = 0.052$). However, when mice were primed with the same strains of RB51 and boosted with proteins Ag85B and ESAT6, a significant level of protection (1 log reduction) compared to the PBS vaccinated group was achieved. The protection levels conferred by this vaccination strategy was similar to that conferred by BCG vaccine. In conclusion, we have shown that recombinant RB51 strains expressing mycobacterial protective antigens result in stimulation of antigen specific immune response without altering the vaccine efficacy in protecting against the more virulent strain of *B. abortus* 2308. These recombinant vaccines could potentially be used to protect against *M. tuberculosis* infection.

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List of Abbreviations

BCG	Bacillus Calmette Guerin
BSL-3	Biosafety Level 3 laboratory
CFU	Colony forming units
CMI	Cell mediated immunity
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immuno Sorbent Assay
Esat-6	Early Secreted Antigenic Target
H1	Hybrid 1
H56	Hybrid 56
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
kDa	Kilodalton
LPS	Lipopolysaccharide
MDR	Multidrug-resistant
MHC	Major Histocompatibility Complex
MTB	Mycobacterium tuberculosis
OADC	Oleic Acid Dextrose Catalse supplement
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel

SOD	CuZnSOD
Th1	T helper 1
Th2	T helper 2
TNF	Tumor necrosis factor
TSA	Tryptic Soy Agar
ug	Microgram
ul	Microliter

Chapter 1

Literature Review

Mycobacterium species and Prevalence

> "If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like. One in seven of all human beings dies from tuberculosis."
>
> –Robert Koch, 1882

With these powerful words Robert Koch, a German physician and scientist, concluded his presentation on the evening of March 24, 1882. In his speech, Koch presented to his audience the discovery of *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis (TB). This was the discovery that earned him the Nobel prize in medicine in 1905. Over 130 years have passed since Koch's discovery, yet high prevalence of TB in human still exist, and to this day TB kills more people than any other disease in the history of humankind.

Mycobacteria are aerobic, acid-alcohol fast, non-sporulating, rod-shaped microorganisms with high GC content. They are members of the genus Actinobacteria, which belong to the order of Actinomycetales [1]. *M. tuberculosis* belongs to a group of phylogenetically closely related bacteria, collectively known as the *Mycobacterium tuberculosis* complex (MTBC) [2, 3]. In addition to *M. tuberculosis* and *M. africanum*, which cause TB exclusively in humans, four other species of MTBC exist, causing TB in a wide range of wild and domestic animal species. These species include *M. bovis*, *M. caprae*, *M. microti* and *M. pinnipedii* [2, 3].

All species within the *Mycobacterium tuberculosis* complex can cause tuberculosis in humans and animals but to a different degree. *M. tuberculosis* is considered the principal causative agent of tuberculosis in humans. However in some countries in Africa, *M. africanum* is just as prevalent as *M. tuberculosis*, causing up to half of human tuberculosis infections in West Africa [4]. Although *M. africanum* has been restricted geographically to countries in West Africa, it has sporadically been identified in other places like Germany, England, France, Spain, and the United States [5-8]. *M. bovis* is the causative agent of bovine tuberculosis, causing more than three billion dollars in agricultural losses worldwide [9]. More importantly, *M. bovis* is

considered a zoonotic pathogen and can be readily transmitted from animals to humans via droplet infection or by food intake including unpasteurized milk [10].

Tuberculosis (TB), caused by *M. tuberculosis,* is one of the most prevalent infectious diseases inflicting humankind. The World Health Organization (WHO) estimates that one third of the world's population, approximately 2.2 billion people, are infected with TB with a mortality of 1.7 million people annually [11, 12]. In 2012, the WHO reported a prevalence of approximately 8.6 million cases of human TB globally. Most of the human cases occurred in Asia (58%) and Africa (27%), whereas smaller proportions of cases occurred in the Middle East (8%), Europe (4%) and the Americas (3%) [13]. The majority of the tuberculosis burden, 80% of the global burden, exists in twenty-two countries. These countries are led by India and China, which together have approximately one-third of the total global TB burden [13]. Due to immigration and travel, tuberculosis can be readily spread around the world, and extremely difficult to eliminate in any single country [14].

In humans, symptoms of active tuberculosis often include coughing, fever, nightly sweats, wasting of the body, and various other symptoms depending on the part of the body affected. Tuberculosis usually affects the lungs;

however, it can also affect other body organs such as the brain, the kidneys or the bones [15]. Usually, the initial screening test for tuberculosis is the tuberculin skin test. A small amount of purified protein derivative from *M. tuberculosis* is injected under the skin of the forearm. The area is visually examined by a health professional after 48-72 hours to determine the result of the test. A positive skin test indicates a type IV hypersensitivity response, meaning an individual may have been exposed to the microorganism but does not necessarily mean an active disease is present. If an individual tests positive, a chest X-ray must be obtained to ascertain whether there is any active disease [16].

Antibiotics against tuberculosis

Streptomycin was the first antibiotic and bactericidal agent shown to be effective against *M. tuberculosis*. It was discovered in 1943 by the 1952 Noble Prize winner in Medicine, Selman Waksman and his student at the time, Albert Schat. Streptomycin was derived from the Actinobacterium *Streptomyces griseus*; however, the mechanism of action of streptomycin was not known at the time of discovery. Its potency was later attributed to inhibition of bacterial protein synthesis by binding to the 30s subunit of the bacterial ribosome [17]. During the same time that Waksman was working

on streptomycin, a Swedish scientist by the name of Jorgen Lehmann was working on another antimicrobial, a synthetic one called para-amino salicylic acid (PAS), a drug that proved to be very potent against *M. tuberculosis*. Despite being in clinical use for over 70 years, the mechanism of action of PAS is still elusive [18]. In a landmark discovery by the UK Medical Research Council, it was shown that the combination of streptomycin and PAS resulted in a superior treatment over either agent alone [19]. This discovery led to the establishment of the first multi-drug therapy approach which combined two or more antimicrobials against tuberculosis infection.

The introduction of isoniazid in 1952 combined with streptomycin and PAS led to what came to be the first triple chemotherapy against tuberculosis, curing many tuberculosis patients in 18–24 months [20, 21]. The introduction of other antibiotics led to refinement of these combination antibiotic regimens. PAS was replaced by ethambutol, a synthetic compound with antituberculosis activity discovered in 1961 [22]. The addition of rifampicin in 1970s and the replacement of streptomycin by pyrazinamide in 1980s led to the development of the short-course chemotherapy. This regimen consists of four antibiotics; ethambutol, isoniazid, pyrazinamide and rifampicin and has a treatment duration of 6-8 months [23].

Although most cases of tuberculosis can be treated successfully by the multidrug combinations of antimicrobials mentioned earlier, the emergence of multidrug-resistant (MDR) strains and the more recent occurrence of totally drug-resistant (TDR) strains of *M. tuberculosis* has made interruption of disease transmission very insufficient and has nearly returned tuberculosis treatment to pre-antibiotic era [24]. Therefore, better approaches for preventing infection and subsequent transmission are needed. These measures could include the use of vaccines that can prevent the establishment of TB infection.

Immunology

Two forms of TB infection exist: active and latent. Among those infected with TB, approximately 90% have the latent form of infection [25]. These patients do not demonstrate clinical signs or symptoms, and their immune system prevents the organism from replicating, however, they never eradicate the pathogen. Although most latently infected persons do not die from TB, patients with a latent infection provide a huge reservoir for active TB in which the disease becomes symptomatic, infectious and lethal [26]. It is estimated that 10% of those with latent TB will develop active disease in their lifetime. Recrudescence of TB is usually associated with failure or

breakdown of the host immune response or other predisposing factors such as host genetics, and environmental factors (concurrent infectious disease including HIV, concurrent metabolic disease such as diabetes, and malnutrition [12, 27]. According to the WHO, one out of four TB deaths is HIV related [3, 11, 28, 29].

Currently, the WHO estimates that each year more than 9 million people develop TB [11]. Disease prevalence is further intensified by the increasing emergence of multidrug-resistant strains (MDR) and the more recent occurrence of totally drug-resistant (TDR) strains of *M. tuberculosis*, which are virtually untreatable [30]. Transmission of TB in humans occurs through inhalation of *M. tuberculosis*-containing droplets of respiratory secretion that are expelled into the air via coughing and sneezing. Upon inhalation and deposition of *M. tuberculosis* into the lungs, the innate immune system responds by recruiting alveolar macrophages, interstitial macrophages, and local dendritic cells [11, 31]. Contrary to the role of macrophages as an effective initial barrier to bacterial infection, macrophages are the main locations for *in vivo* persistence of *M. tuberculosis*. The bacterium is able to persist inside those professional phagocytes by implementing several survival strategies against conventional phagocytic destruction [32].

Professional phagocytes such as macrophages and dendritic cells form the cellular arm of the innate immune system. The primary role of these cells is to discriminate non-self (pathogens) from self, and to clear such pathogens through phagocytosis [33]. Effective clearance of potential pathogens requires two components; pathogen engulfment and phagosomal maturation. Upon engulfment by phagocytic cells, the pathogen is trapped in a membrane-bound intracytoplasmic phagosome containing the pathogen in extracellular fluid. These phagosomes are then fused with a digestive enzyme-containing lysosome in a process called phagosomal maturation leading to the formation of a phagolysosome [32]. During maturation, several innate antimicrobial features are invoked that are lethal to many pathogens. These include acidification, deployment of reactive oxygen and nitrogen species as well as antimicrobial peptides. This sequence of events leads to ingestion and total destruction of the pathogen [34]. However, with TB infection, the bacteria is able to persist inside those professional phagocytes not only by arresting phagosome maturation but also by escaping the phagosome. The process by which *M. tuberculosis* arrests maturation of phagosome remains to be fully understood. However, survival in phagocytes has been partially attributed to the urease produced by *M. tuberculosis* which produces ammonia that blocks vacuole acidification [35] and the

hydrophobic nature of the bacterial surface which influences phagolysosomal membrane fusion [36]. *M. tuberculosis* is also capable of modulating other macrophage defenses to promote its survival including: inhibition of apoptosis and by blocking of MHC antigen processing and presentation, which is used to promote an adaptive immune response [37-39].

Although the correlation of protection in TB is not yet resolved, it has been well established that protection against facultative intracellular bacteria such as *Mycobacterium* requires cell-mediated immunity (CMI) [40]. Many types of T-lymphocytes including $CD4^+$ helper T-lymphocytes, $CD8^+$ cytotoxic T-lymphocytes and γ/δ T-lymphocytes play a role in host defense against TB. However, studies of the immune response to *M. tuberculosis* in humans and animal models have shown that $CD4^+$ is undoubtedly the major effector cell in CMI [41, 42]. During TB infection, $CD4^+$ T-cells differentiate into Th1 $CD4^+$ T-cells which in turn mediate immune responses to intracellular pathogens induce production of interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-12 (IL-12), which collectively upregulate innate and adaptive CMI responses. In addition to $CD4^+$ T-cells, $CD8^+$ T-cells also contribute to optimal immunity and protection against TB through cytotoxic activity [43, 44]. However, the mechanisms underlying $CD8^+$ T-cell activation in TB is poorly understood [11]. The role of these

cells has been demonstrated by the observation that TB infection of $CD4^+$ and $CD8^+$ knockout mouse models leads to increased bacterial burden and shortened survival times [45]. These findings demonstrate that control of TB infection is dependent on the cellular immune response. As a result, an effective vaccine against TB will need to elicit and activate the cellular arm of the immune system.

Vaccines against Tuberculosis

Bacille Calmette Gue´rin (BCG) vaccine is an attenuated strain of *M. bovis* that was isolated in 1921 by the two scientists, Albert Calmette and Camille Guérin [46, 47]. BCG was derived by serially passing the virulent *M. bovis* strain on *ox-bile* medium for 230 times in the laboratory between 1908 and 1921. This attenuation of the virulent *M. bovis* led to self-limiting infection in humans as well as partial resistance to reinfection with *M. tuberculosis* and *M. bovis* [47]. To date, BCG continues to be one of the most widely used human vaccines, administered to approximately 100 million infants annually worldwide [48]. Although used for almost a century, the use of BCG remains controversial, with known variations existing in BCG substrains, vaccine efficacy, and health policies and practices across the world [49].

Clinical studies have shown childhood vaccination with BCG to be protective against disseminating and meningeal forms of TB. However, the efficacy of BCG against pulmonary TB in adults has been variable and inconsistent (0-80%) [12, 50]. Lack of effectiveness to pulmonary TB may be due to increasing attenuation of the BCG strains [46], waning of protective efficacy over time [51], and genetic differences in strains of BCG as well as differences in the genetic backgrounds of the tested populations [52]. Additionally, people vaccinated with BCG usually test positive to the tuberculin skin test (TST); thus complicating interpretation of the diagnostic test most widely used worldwide [53]. The variation in the efficacy of BCG against tuberculosis in adults as well as the complications associated with its use in diagnostic tests have lead to different vaccination policies worldwide. For example, the United Kingdom implements and supports universal BCG vaccination programs, while other countries like the United States and Canada do not advocate for BCG usage countrywide and only recommend it for high-risk groups [49]. In countries that undergo mass vaccination programs, BCG is usually administered to children at birth or within the first year, and a booster or a third vaccination is administered by the age of 15 [49]. Clearly, the lack of an effective vaccine, increased emergence of MDR strains of *M. tuberculosis*, and the highly infectious nature of the disease

warrant development of a more effective vaccine for the control of tuberculosis in humans.

The pandemic nature of TB combined with the poor efficacy of the current vaccine and the rising incidence of multidrug resistant strains encouraged the development of new vaccines against TB. Historically, the development of new TB vaccines has been focused on two major strategies; improved recombinant BCG and subunit vaccines [11, 54, 55]. One of the recombinant BCG vaccines that has advanced to clinical trials is rBCG30. This recombinant vaccine was engineered by overexpressing the 30-kDa mycolyl transferase of *M. tuberculosis* (Ag85B). Upon aerosol challenge with a high dose of the highly virulent strain of *M. tuberculosis,* immunized guinea pigs with rBCG30 had 0.5 log and 1 log fewer *M. tuberculosis* bacilli in their lungs and spleens, respectively, than animals immunized with the parental BCG vaccine [56, 57]. This vaccine has been shown to be immunogenic in humans and at the present time has passed phase I clinical safety trial [58, 59].

VPM1002 is another recombinant form of BCG that overexpresses listeriolysin, a protein derived from *Listeria monocytogenes*. Expression of listeriolysin in *L. monocytogenes* enables the microorganism to escape from

the phagosome and translocate to the cytosol of infected host cells. BCG induces weak apoptosis and CD8+ T cell stimulation, however, leakage of the VPM1002 into the cytosol leads to improved exposure of mycobacterial antigens to the MHC Class-I pathway, which results in stronger induction of apoptosis and better stimulation of CD8+ T cells [60] which contribute to protection against TB infection [61]. In a mouse efficacy study, VPM1002 strain was shown to be significantly more efficacious in inducing protective immunity against tuberculosis than parental BCG. More importantly, VPM1002 induced superior protection not only against laboratory strains of *M. tuberculosis*, but also against the more virulent clinical isolates of *M. tuberculosis*, including isolates in the Beijing family [62]. In a human clinical trial, VPM1002 was shown to stimulate IFN-γ-producing and multifunctional T cells, as well as antibody-producing B cells in BCG-naïve and BCG-immune individuals. The safety and immunogenicity of VPM1002 was comparable to that of parental BCG strain [63]. A literature search suggests that recombinant BCG strains have been found safe in clinical trial, but the protective efficacy of these vaccine candidates against TB was not substantially greater than protection induced by BCG [11, 64, 65]. Additionally, those recombinant forms of BCG do not address the issue of safety in immunocompromised individuals, in whom it can cause serious

disseminated disease and even death, or the complications associated with BCG usage in the tuberculin skin test [66].

In the case of subunit vaccines, the accelerated identification of novel antigens has led to the identification of defined antigens against TB. Culture filtrates have attracted particular interest as a source of protective antigens and have been evaluated in various animal models of TB [67-69]. Hybrid 1 (H1) candidate vaccine is one of the most prominent subunit vaccines that was produced by fusing two immunodominant antigens of *M. tuberculosis*, Ag85B and ESAT6. In preclinical trials, this subunit vaccine resulted in prolonged survival in the extremely susceptible guinea pig model [70], similar protection to BCG in mouse models [71] and protection in a non-human primate model [72]. In clinical trials, H1 was shown to be safe and highly immunogenic in human beings [73].

Hybrid 56 (H56) is another subunit vaccine that was produced by fusing hybrid 1 (Ag85B and ESAT6) along with Rv2660c, a latency-associated protein. In preclinical trials, vaccination of mice with H56 resulted in protection similar to that afforded by BCG. However, the protective immunity induced by H56 was characterized by a more efficient containment of late-stage infection than the Hybrid 1 or BCG vaccines [74].

In non-human primates boosting with H56/IC31 after priming with BCG resulted in efficient containment of *M. tuberculosis* infection, reduced rates of clinical disease, and improved survival of the animals compared to BCG alone [75]. A literature search of potential subunit vaccines against tuberculosis shows that in general, the ability of these subunit vaccines to induce a protective and long-lasting immune response by themselves limited. Therefore, current views are that subunit vaccines may be effective as booster vaccines after priming with BCG or recombinant BCG strains [11, 52, 76]. Currently, other mechanisms of vaccine development strategies are being investigated. These include DNA vaccines, attenuated *M. tuberculosis* strains, and other non-microbial live vectors carrying TB antigens [55, 64, 77-79]. Although these approaches utilize different platforms for presenting *M. tuberculosis* antigens to the immune system, they all target the same type of immune response; cell-mediated immunity.

M. tuberculosis protective antigens

Antigen 85B

Antigen 85B (Ag85B) is an immunodominant protein antigen secreted by all mycobacterial species [80]. Ag85B (30 kDa) belongs to the Ag85 complex, which is a family of three structurally related fibronectin-binding

proteins (Ag85A, Ag85B and Ag85C) with mycolyl- transferase activity that are involved in the final stages of cell wall assembly [81]. Ag85B protein is not only the major secretory protein of *M. tuberculosis* in broth culture, but it is also one of the major proteins expressed in human macrophages. It is a major stimulator of T-cell proliferation and IFN-γ production in most healthy individuals infected with *M. tuberculosis* [82-85]. Immunization with a DNA vaccine encoding Ag85B can stimulate strong cell-mediated immune response and confer significant protection in mice against experimental infection with *M. tuberculosis* [85].

Early secretory antigenic target 6

Early secretory antigenic target 6 (ESAT6) is a protein that is encoded by the region of difference 1 (RD1) of the *M. tuberculosis* genome [86]. RD1 has been shown to be a major virulence factor involved with membrane-lysing activity [87, 88]. Although the exact function of ESAT6 has not been determined, studies have shown that deletion of the ESAT6 protein results in abrogation of the necrosis-inducing effect of *M. tuberculosis* on human monocyte-derived macrophages, suggesting that ESAT6 is involved with causing necrosis [89]. ESAT6 is a T-cell antigen that is strongly recognized in the first phase of infection and has demonstrated protective efficacy in

animal models as a subunit [90], DNA [91], and recombinant BCG vaccines [92].

Rv2660c

Rv2660c is a newly recognized antigen of unknown function that was first reported in a gene expression profiling study by Betts and colleagues [93]. Expression of Rv2660c is increased 100-300 fold in nutrient-starved cultures, making it the most strongly up-regulated of all nutrient starvation–induced genes identified under these *in vitro* conditions. In another gene expression profiling study in a mouse model, it was found that Rv2660c was expressed at high levels during early and late stages of infection [74]. More importantly, Govender and colleagues reported that Rv2660c was preferentially recognized by patients with latent TB as compared to patients with active TB disease [94]. These findings suggest that Rv2660c is involved in latency and may be a promising vaccine candidate for targeting latent TB infection.

Brucella abortus

Brucella spp. are Gram-negative, facultative intracellular bacteria that cause zoonotic disease of brucellosis in a wide range of animals and humans [95]. Based on differences in host preference and phenotypic characteristics, the

genus *Brucella* is divided into six species; *B. abortus*, *B. melitensis*, *B. canis*, *B. suis*, *B. ovis* , *B. ceti, B. microti, B. penipedialis* and *B. neotomae,* with the first five being pathogenic to humans [96, 97]. In contrast to other pathogenic bacteria, *Brucella* spp. lack classical virulence factors such as invasive proteases, exotoxins, capsules, fimbriae, pilli, plasmid lysogenic phages and drug resistance [98, 99]. Rather, *Brucella* employs other strategies to evade the innate immune system. These strategies include a type IV secretion system, which is induced during phagocytosis by macrophages and leads to translocation of effector proteins into the host cytosol and eventually to inhibition of the phagosome-lysosome fusion [95]. *Brucella* also prevents apoptosis of macrophages by suppressing mitochondrial gene expression involved in cytochrome C release, reactive oxygen species production, mitochondrial membrane permeability, and preventing activation of caspase cascades, thus creating a conducive environment for replication and persistence [98]. The lipopolysaccharide (LPS) structural component of the *Brucella* outer membrane has also been reported to be a virulence factor [100, 101]. LPS of *Brucella* is composed of three main domains; the lipid A, the core oligosaccharide, and the O side chain [102]. Smooth strains of *Brucella* spp. contain the O side chain, whereas the rough strains lack the O side chain. In *B. abortus* the smooth

LPS is considered a virulence factor as well as the immunodominant antigen. Rough mutants that were derived from smooth virulent strains are less virulent than the wild type [103] and do not induce antibodies against LPS, thus they do not interfere with interpretation of serological tests

RB51 vaccine as vector

B. abortus vaccine strain RB51 is a stable rough mutant that was derived from the standard virulent strain 2308 though repeated passage on growth medium supplemented with varying concentrations of rifampin or penicillin [104]. It was thought that this *in vitro* passage of strain RB51 resulted in a mutation in one of the genes encoding one of the Lipopolysacchride (LPS) biosynthetic pathway; consequently there is a loss of LPS, a major antigenic and toxic component of the standard virulent strain 2308. One of these mutations has been traced to the presence of an IS711 element in the *wboA* gene responsible for a glycosyltransferase involved in the synthesis of the O-chain [105]. However, other mutations involving LPS synthetic pathway have been described. As studies have shown that complementing RB51 with the *wboA* gene does not change the rough phenotype of RB51, indicating that other mutations in the LPS synthetic and trafficking pathways have occurred [105]. This loss of the LPS endotoxicity along with the antigenic

properties of strain RB51 have made it a prime vaccine candidate to protect cattle against brucellosis.

For the last two decades strain RB51 has been the official vaccine for cattle brucellosis in the United States and elsewhere. [104]. Isolated in 1991, strain RB51 has been shown to induce protection against *B. abortus* 2308 challenge primarily through cell-mediated immunity (Th1) [106]. More importantly, studies have shown that immunization of mice with recombinant strains of RB51 expressing heterologous proteins leads to a Th1 type of immune responses specific to the expressed protein without altering its vaccine efficacy against *B. abortus* challenge [105, 107, 108]. These findings provide a rationale for the use of strain RB51 as a vector for the delivery of protective antigens of *M. tuberculosis*, in which Th1 type immune responses are essential for protection. The feasibility of expressing foreign proteins in strain RB51 makes it a testable model for the development of a live, attenuated bivalent vaccine against brucellosis and tuberculosis infections in humans and animals. However, strain RB51 has been shown to cause illness in humans [109] and further refinement would be needed before it may be introduced as human vaccine. One way to make strain RB51 safer is through gamma irradiation. Sanakkayala and colleagues have shown that exposing strain RB51 to gamma irradiation leads to an

inactivated live vaccine (non-viable), that is still metabolically active. This inactivation does not interfere with the induction of Th1-type immune responses or the protective efficacy against *B. abortus* challenge [110].

Brucellosis and Tuberculosis-drawing the parallel

Both tuberculosis and brucellosis are infections that are caused by intracellular bacterial pathogens that require strong cell-mediated immunity or Th1 type of immune response for their elimination from the host. Although there are no studies linking the two infections, some similarities exist. Both organisms live and replicate inside the phagosomal vesicles of macrophages and survive inside those macrophages by inhibiting phagosome-lysosome fusion. As a result both infections require activation of cytoxic T lymphocytes for microorganism clearance. Also the Th1 cytokine profile needed to clear both infections is similar; both infections require secretion of interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). Since strain RB51 is a potent inducer of such immune response, overexpression of *M. tuberculosis* antigens in RB51 could potentially lead a similar immune response against tuberculosis infection.

Objective of the Dissertation

Tuberculosis remains arguably one of the most *common* infectious diseases worldwide, with nine million new infections occurring every year, and the leading cause of ~2 million deaths in the world from a single pathogen [111]. The rising prevalence of multidrug-resistant (MDR) strains of *M. tuberculosis*, coupled with the highly infectious nature of the disease constitutes a great public health burden on the society. However, this burden might be ameliorated by an effective vaccine. The attenuated *M. bovis* BCG vaccine is currently the only TB vaccine approved for human use, but its efficacy remains controversial [52]. Therefore, there is a need for a vaccine that would prevent the initial establishment of TB infection and, subsequently, prevent transmission of the disease between healthy individuals. The objective of this dissertation is to develop and test the efficacy of the *B. abortus* vaccine strain RB51 as a platform for expression of *M. tuberculosis* antigens (Ag85B, ESAT6 and Rv2660c) and induction of protective immune response against *M. tuberculosis* and *B. abortus* challenge in a mouse model. Two types of expression vectors are utilized in this study; one with a signal sequence for secreting *M. tuberculosis* antigens and another for cytoplasmic expression. *The efficacy of the recombinant RB51 vaccine candidates* was then tested in mice in a vaccination challenge

study. We predicted that immunization of mice would induce a strong cell-mediated immunity, the type of immune response needed to protect against *M. tuberculosis*. The data generated from this study could potentially be used to encourage further refinement of strain RB51 so that it can be used as a bivalent vaccine against brucellosis and tuberculosis infections in humans.

Overall goal

To determine the efficacy of recombinant strain RB51 carrying *M. tuberculosis* protective antigens in inducing protective immunity against *M. tuberculosis* and *B. abortus* infections.

Overall hypothesis

Immunization with the recombinant RB51 strains expressing *M. tuberculosis* antigens will induce a cell-mediated immune response against *B. abortus* and *M. tuberculosis* thereby provide protection against a challenge in mice. Achieving the following aims will test the proposed hypothesis:

Aim 1: Construction of plasmid vectors for expression and secretion of heterologous proteins in strain RB51.

Aim 2: Expression and secretion of the *M. tuberculosis* protective antigens (Ag85B, ESAT6, and RV2660c) in the *B. abortus* vaccine strain RB51.

Aim 3: Determining the protective and immunogenic efficacy of each candidate vaccine in a BALB/c mouse challenge study.

Chapter 2

Development of vectors for expression and secretion of heterologous proteins in *B. abortus* strain RB51

Abstract:

Strain RB51 is the official vaccine against cattle brucellosis in the United States and other countries. Protection conferred by strain RB51 vaccination is due to induction of cell-mediated immunity, more specifically though production of IFN-γ. Several studies have utilized strain RB51^*leuB* as a platform for expression of protective antigens of other intracellular pathogens in which Th1 type immune response or cell-mediated immunity is essential for protection. In this study, we have constructed two modified expression vectors: one for cytoplasmic expression, pNS4TrcD, and another for expression and secretion, pNS4TrcD-FlgE. Using GFP as a reporter protein we were able to detect GFP in the cytoplasm of RB51-pNS4TrcD-GFP as well as in the supernatant of RB51-pNS4TrcD-FlgE-GFP. These two modified will allow for expression and secretion of other heterologous protein in strain RB51^*leuB*.

Introduction:

B. abortus strain RB51 is a stable rough mutant that was derived from the wildtype virulent strain *B. abortus* 2308 [1]. This strain is currently the official USDA approved vaccine against cattle brucellosis in the United States and elsewhere. The safety and protective efficacy of strain RB51 have been well demonstrated under laboratory and field conditions [2, 3]. Protection afforded by strain RB51 vaccination is due to induction of cell-mediated immunity, more specifically though production of IFN-γ [4-6]. Multiple studies have exploited the advantageous vaccinal qualities of strain RB51 as a vector for the delivery of protective antigens of other intracellular pathogens in which Th1 type immune response or cell-mediated immunity is essential for protection [4-9]. However, these studies were not able to utilize strain RB51 to its full potential. This was due to the weak expression of foreign antigens, which was a result of using weak promoters; the site of antigen delivery, as most of these studies were focused on cytoplasmic expression as opposed to periplasmic expression or secretion; as well as the use of unstable drug resistant expression vectors i.e plasmid, which are usually lost in the absence of selection pressure. The objective of this chapter is to construct stable expression vectors that are capable of high-level expression and secretion of heterologous proteins by strain RB51.

Traditionally, plasmid-based expression systems have been utilized to over-express heterologous proteins in strain RB51 [4-7, 10-13]. However, these expression vectors usually code for antibiotic resistance markers and the Food and Drug Administration strongly discourages and strictly regulates the introduction of such antibiotic resistance genes into a live attenuated vaccine [14]. Also, such expression systems are unstable in the absence of antibiotic selection pressure. To overcome this hurdle, an environmentally safe leucine auxotroph strain of RB51 (RB51*leuB*$^{-}$) was created to over-express antigens without using antibiotic resistance marked plasmids [15]. This auxotroph strain can be complemented with pNS4 expression vector carrying the wild-type *leuB* gene. This complementation allows for survival of the strain in leucine-deficient minimal medium and under nutrient-limiting conditions *in vivo* [16], thus providing selective pressure for plasmid maintenance without using antibiotic selection markers. Moreover, the stability of this expression vector has been documented *in vitro* and *in vivo* [15]. However, the promoter (*groE*) utilized in pNS4 to over-express heterologous protein in RB51*leuB*$^{-}$ allowed for moderate antigen expression [13]; a better promoter is essential for strong expression. Therefore, the purpose of this chapter is to construct expression vectors that are capable of high expression levels of heterologous proteins in strain RB51*leuB*$^{-}$. This

was accomplished by replacing the *groE* with the *trcD* promoter, a semi-synthetic promoter that has been shown to have the strongest transcriptional activity in *Brucella* [13]. The feasibility of secreting heterologous antigens in strain RB51 was also explored. Secreted antigens of *M. tuberculosis* such as Ag85B and ESAT6 are central to the induction of protective immunity against tuberculosis infection [17, 18] and construction of expression vectors capable of secreting these antigens in strain RB51*leuB*$^{-}$ was examined.

Brucella species have a complete and functional type IV secretion system [19]. This secretion apparatus in *Brucella* is typified by the *virB* operon, encoding 12 membrane-associated *proteins* that are postulated to form the macromolecular transfer mechanism [20]. The mechanism of assembly and the complete set of macromolecules secreted by *vir*B in *Brucella* are still unknown; however, the *virB* region has been shown to be essential for intracellular survival and multiplication in the three most pathogenic strains of *Brucella*: *B. suis*, *B. abortus* and *B. melitensis* [21-24]. Studies have shown that *vir*B mutant strains lose their ability to survive and multiply intracellularly and, as a result, become attenuated [25].

Brucella spp. utilize protein members of the conjugative type IV secretion system, termed coupling proteins (CPs). These proteins interact directly with

secreted substrates, presumably through binding secretion signal peptides, and mediate the transfer of these substrates to specific subunits of the secretion channel [26]. Therefore we hypothesize that secretion of heterologous proteins in strain RB51 can be achieved by utilizing signal sequences of membrane associated *Brucella* proteins or other proteins that are secreted into culture supernatants with well-defined signal peptides.

Materials and Methods:

Recombinant DNA methods (DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis) were performed according to standard techniques [27]. The polymerase chain reaction (PCR) was performed using Platinum PCR SuperMix High Fidelity (Invitrogen, USA) and a Gradient Mastercycler® (Eppendorf). Oligonucleotides were purchased from Sigma-Genosys (Sigma-Aldrich, USA). Restriction and modification enzymes were purchased from Promega. The QIAprep Spin Miniprep Kit (QIAGEN, USA) was used for plasmid extractions and the QIAGEN PCR cleanup kit was used for restriction enzyme removal and DNA gel extraction.

Design and synthesis of signal sequences:

Several *Brucella* proteins were analyzed for their subcellular location and the presence of predicted signal sequences using the Signal PV1.1 and PSORT-B prediction softwares (Table 2.1) [28]. Three DNA sequences (*trcD-flgE, trcD-CoxA* and *trcD-sucB*) each consisting of a *trcD* promoter, a signal sequence, a His-tag, and two restriction sites (*Sal*I and *BamH*I) for directional cloning were designed and synthesized by Genscript Inc., USA (Figure 2.1).

Replacement of *groE* promoter with the synthesized promoters

The starting point for construction of the expression vectors was the pNS4GroE plasmid. The *groE* promoter was excised from pNS4GroE expression plasmid with *Sal*I and *BamH*I restriction enzymes. Synthesized promoters along with their signal sequences were digested with *Sal*I and *BamH*I and ligated into the promoterless pNS4 (Figure 2.3 & 2.4). The ligated vectors were transformed into *E. coli HBl01leuB*$^{-}$ via electroporation to create pNS4TrcD, pNS4TrcD-FlgE, pNS4TrcD-CoxA and pNS4TrcD-SucB expression plasmids. Successful transformants were screened for the presence of the synthesized promoter by restriction digestion and DNA sequencing of inserts using the primers shown in (Table 2.2).

Cloning of GFP into expression vectors

The gene coding for green fluorescent protein (GFP) was amplified via polymerase chain reaction (PCR) using pNSTrcD-*gfp* [29] as a template and the primers listed in (Table 2.2)The PCR amplicon was gel purified, digested with *Bgl*II and *Xba*I and cloned into the new expression vectors, which were previously digested with *BamH*I and *Xba*I, to create pNS4TrcD-GFP, pNS4TrcD-FlgE-GFP, pNS4TrcD-CoxA-GFP and pNS4TrcD-SucB-GFP (Figure 2.3). Following transformation into strain RB51*leuB*-, the transformants were confirmed by restriction digestion and DNA sequencing of inserts.

Testing promoter activity of the constructed vectors using GFP as a reporter protein in RB51*leuB*$^{-}$

The newly constructed plasmids carrying synthesized promoters along with *gfp* were electroporated into strain RB51*leuB*- as described earlier [30]. Transformants were selected by plating on leucine-deficient *Brucella* minimal medium (BMM*leu*$^{-}$) plates. Colonies of strain RB51*leuB*$^{-}$ harboring the vectors and expressing GFP appeared as green fluorescent colonies when observed under UV light. These transformants were later screened for the presence of the expression vectors by plasmid extraction and restriction

digestion.

Evaluation of plasmid stability *in vitro*

To evaluate the stability of the newly constructed plasmids expressing GFP, three random colonies from each construct were subcultured onto either non-selective-enriched medium (TSA) or selective medium (BMM*leu*-). The subcultures were evaluated for GFP expression under UV light and, subsequently, subcultured onto new BMM*leu*- agar plates every 4 days until fluorescence was no longer observed.

Preparation of the protein extract and western blotting

Expression and secretion of GFP was also confirmed by western blotting using anti-His antibodies. Briefly, cultures of strain RB51*leuB*$^{-}$ carrying expression plasmids along with *gfp*, were grown in liquid BMM*leu*- to mid-log phase (OD 100). For cytoplasmic GFP expression, 100 µL of each culture was pelleted for 5 minutes in a microcentrifuge tube at 12,000 x G. The pellet was then lysed with 5% β-mercaptoethanol in the presence of 10 mM Tris-base (pH 8.1) and heated in a water bath at 95 ºC for 10 minutes. For secreted GFP, 1 ml of each culture was pelleted for 15 minutes in a microcentrifuge tube at 12,000 x G. The collected supernatant was pelleted again to remove residual bacterial cells. Supernatants were then precipitated

overnight at 4 C° in 10% trichloroacetic acid (TCA), centrifuged at 12,000 x G for 15 minutes and washed twice with pre-chilled acetone to remove residual TCA. Air-dried pellets were dissolved in 40 µl of SDS-PAGE sample buffer. Western Blot analysis was performed on proteins transferred onto nitrocellulose membranes and probed with 1:3000 dilutions of mouse IgG2a anti-His, horseradish peroxidase (HRP) conjugated primary antibodies.

Results:

Bioinformatics analysis

Six *Brucella* proteins were analyzed with PSORT-B and SignalP V1.1 programs [28] in order to predict the subcellular location of proteins and the presence of signal peptides (Table 2.2). All six proteins analyzed were predicted signal sequence within the N-terminal 50 to 70 amino acids using the program SignalP V1.1. Consistent with our literature search, 2 of the 6 proteins analyzed, BP26 and SOD, were determined to be located in the periplasmic space of *B. abortus* [31, 32]. CoxA and SucB proteins were shown to be associated with the inner and outer membrane respectively. PPIase was shown to be a cytoplasmic protein while FlgE was shown to be an extracellular protein as well as a subunit component of the flagellum.

Promoter construction and replacement

Figure 2.1 depicts the *trcD* promoter along with the different signal sequences. A His-tag was engineered downstream of the signal sequences for protein detection and purification. Two restriction sites, *Sal*I and *BamH*I were also added to synthesized promoters for directional cloning. The sequences were synthesized by Genscript, USA and cloned into pUC57 (Genscript, USA). The promoters were then cloned into the promoterless pNS4 using *Sal*I and *BamH*I restriction sites to create the four expression vectors. Upon construction of the expression vectors, the gene encoding green fluorescent protein was cloned in using *Bgl*II and *Xba*I restriction sites (Figure 2.4 depicts the cloning strategy for one of the expression vectors).

Activity of synthesized promoters

A comparison was made between the relative levels of GFP expression under the newly constructed promoters and the original *groE* promoter. All four constructed promoters were able to allow for expression of GFP in strain RB51*leuB*$^{-}$. Upon examination of individual colonies expressing GFP under UV light, it was apparent that expression of GFP under all four synthesized *trcD* promoters was stronger than that under *groE*, the original

promoter. More importantly, the expression vectors were maintained for more than 25 passages on selective media. However, when grown on non-selective enriched media (TSA), the same recombinant strains were stable up to 9 passages, as shown by GFP expression revealed under UV light.

Secretion of heterologous proteins

GFP was used to evaluate the ability of the signal sequences to traffic expressed heterologous proteins extracellularly. Two methods were utilized to monitor secretion of GFP: visualization of culture supernatant under UV light and precipitation of proteins in culture supernatant and subsequent Western blotting. Culture supernatants of the three recombinant strains along with 2 proper controls, non-GFP expressing RB51*leuB*$^-$ and cytoplasmic GFP expressing RB51*leuB*$^-$ strains, were visualized under UV light for the presence of green fluorescence. The *trcD-flgE* promoter was the only promoter capable of expressing GFP that became extracellular. The *trcD-coxA* and *trcD-sucB* were able to express GFP, however their expression was maintained in the cytoplasm. No GFP was present in the supernatant of the control (RB51TrcD-GFP).

Discussion:

Vectors for expression and secretion of heterologous proteins extracellularly in the *B. abortus* vaccine strain RB51*leuB*$^{-}$ were created. To do so, the *trcD* promoter with or without the signal sequences of three *Brucella* proteins: flagellar hook protein (FlgE), dihydrolipoamide succinyl transferase (SucB), and dihydrolipoamide succinyl transferase(CoxI) was synthesized and cloned into the promoterless pNS4 to create pNS4TrcD, pNS4-TrcD-FlgE, pNS4-TrcD-CoxA and pNS4-TrcD-SucB (Figure 2.5). Expression of GFP under the newly synthesized promoters was stronger than that under *groE*, the original promoter. This was consistent with the findings of Seleem and colleagues [13], where it was shown that expression of *lacZ* under the *trcD* promoter was 2-3 times stronger than that under the *groE* promoter in *B. suis*. This strength in expression was attributed to the placement of an A tract, an A+T rich upstream element, between the –38 and the –59 of the core *trc* promoter [33]. This enhancement in expression appeared to be due to the ability of the A tract to provide a binding site for the RNA polymerase [33, 34]. More importantly, the strong expression of GFP by the newly constructed vectors did not compromise the stability of the expression vectors when grown on selective medium (BMM*leu*-). However in the presence of enriched medium or non-selective medium, the recombinant

strains were stable for up to 9 passages. This was consistent with plasmid stability data reported by Rajasekaran and colleagues [15].

A series of signal sequences were synthesized downstream of the *trcD* promoter intended to direct heterologous proteins in strain RB51 extracellularly. All three signal sequences were tagged with a His-tag for protein detection and purification. Only the signal sequence of FlgE was able to direct GFP extracellularly. This was evident by the presence of green fluorescent culture supernatant under UV light as well as the detection of a 31 kDa band corresponding to the size of GFP along with the signal sequence (Figure 2.6). Neither signal sequence of *SucB* or *Cox*I was able to direct GFP extracellularly. This is consistent with the bioinformatic analysis, which indicated that both proteins were associated with inner and outer membranes.

Although *Brucella* species possess open reading frames encoding the flagellar apparatus, phenotypically they are considered nonmotile bacteria [35]. However, this apparatus has been shown as a virulence factor. This was first reported by Fretin and colleagues [36], where they evaluated the effect of mutating different genes coding for various parts of the flagellar structure: MS ring, P ring, motor protein, secretion apparatus, hook and

filament. Upon challenging mice with these mutants, none of them were able to establish a chronic infection like the parent strain *B. melitensis* 16M. The authors attributed this attenuation to the inability of these mutants to secrete virulence factors.

In summary, we have exploited the feasibility of high-level expression and secretion of heterologous proteins in strain RB51*leuB*$^{-}$. The replacement of the *groE* promoter with *trcD* appeared to have achieved a relatively higher level of expression. The fusion of the FlgE signal sequence downstream of *trcD* promoter led to secretion of expressed proteins extracellularly. The expression vectors constructed in this current study proved to be easily transformed into and stably maintained in strain RB51*leuB*$^{-}$.

Protein	Protein description	MW kDa	Location	Signal peptide
FlgE	Flagellar hook protein	40.8	Extracellular	Yes
CoxA	Cytochrome c oxidase subunit 1	41.8	Inner membrane	Yes
PPIase	Trigger factor	53.8	Cytoplasm	No
SOD	Copper/Zinc superoxide dismutase	18.1	Periplasmic space	Yes
Bp26	Uncharacterized protein	26.5	Periplasmic space	Yes
SucB	Dihydrolipoamide succinyl transferase	42.8	Outer membrane	Yes

Table 2.1: List of *Brucella* proteins analyzed using Signal PV1.1 and PSORT-B prediction softwares. Signal peptide presence was annotated by PSORTb v3.0.2

Primer ID	Primer Sequence
trcD-Forward	GTCGACCAGAAAAAA
flgE-Reverse	TACGCTGCCGGAATT
sucB-Reverse	GTTCCAGTTCCACCA
*cox*I-Reverse	AAGCGCGCCGCCGATGAT
gfp-Forward	GGAGATCTATGAGTAAAGGAGA
gfp-Reverse	GGTCTAGATTATTTGTAGAGCTCAT

Table 2.2: PCR primers used to confirm the presence of synthesized promoters and to generate *gfp* for subsequent cloning

TrcD-*CoxA*:

GTCGACCAGAAAAAAGATCAAAAAAATTTGACAATTAATCATCCGGCTCGTA
TAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCGCCGC
TGAGAAAAAGCGAAGCGGCACTGCTCTTTAACAATTTATCAGACAATCTGTG
TGGGCACTCGACCGGAATTATCGATTAACTTTATTATTAAAAATTAAAGAGGT
ATATATTAATGTATCGATTAAATAAGGAGGAATAAACCATGGCTGGCACAGC
AGCTCACGAGCATGGTGCCCACGACGACCACAAGCCGCATGGCTGGGTTCGT
TGGGTATACTCGACCAATCATAAAGACATCGGTACCCTGTACCTGATTTTTGC
AATCATCGCCGGCATCATCGGCGGCGCGCTTCATCATCATCATCATCATGGTG
GATCC

TrcD-*SucB*:

GTCGACCAGAAAAAAGATCAAAAAAATTTGACAATTAATCATCCGGCTCGTA
TAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCGCCGC
TGAGAAAAAGCGAAGCGGCACTGCTCTTTAACAATTTATCAGACAATCTGTG
TGGGCACTCGACCGGAATTATCGATTAACTTTATTATTAAAAATTAAAGAGGT
ATATATTAATGTATCGATTAAATAAGGAGGAATAAACCATGGCTACCGAAAT
TCGCGTTCCCACGCTTGGGGAGTCCGTTACCGAGGCAACCATCGGAAAGTGG
TTCAAGAAGGCTGGTGAAGCCATTGCTGTCGATGAGCCGCTGGTGGAACTGG
AACATCATCATCATCATCATGGTGGATCC

TrcD-*FlgE*:

GTCGACCAGAAAAAAGATCAAAAAAATTTGACAATTAATCATCCGGCTCGTA
TAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCGCCGC
TGAGAAAAAGCGAAGCGGCACTGCTCTTTAACAATTTATCAGACAATCTGTG
TGGGCACTCGACCGGAATTATCGATTAACTTTATTATTAAAAATTAAAGAGGT
ATATATTAATGTATCGATTAAATAAGGAGGAATAAACCATGAGCCTCTACGG
TATGATGCGGACCGGTGTTTCGGGAATGAATGCTCAGGCGAACCGTTTGAGC
ACAGTTGCGGATAATATCGCAAATGCAAGCACGGTCGGTTACAAGCGCGCGG
AAACGCAATTCTCCTCGCTTGTCCTGCCCAGCACTGCCGGACAATATAATTCC
GGCAGCGTACATCATCATCATCATCATGGTGGATCC

Figure 2.1: Synthesized *trcD* promoters with the signal sequences of three *Brucella* proteins. Sequences were color-coded. *Sal*I restriction site (Red), *trcD* promoter (Gray), signal sequence (Green), His-tag (Pink) and *BamH*I restriction site (Yellow).

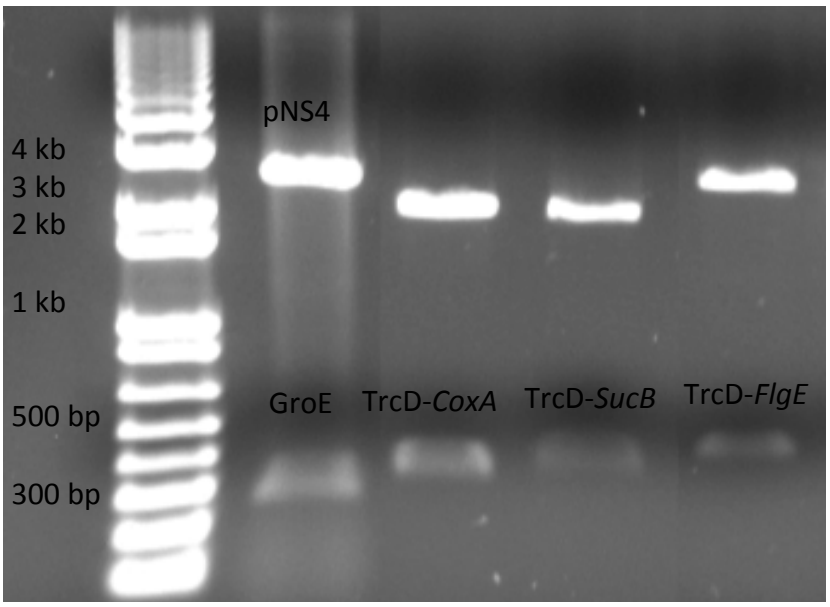

Figure 2.2: Digestion of the three synthesized promoters with *Sal*I and *BamH*I for subsequent cloning into the promoterless pNS4.

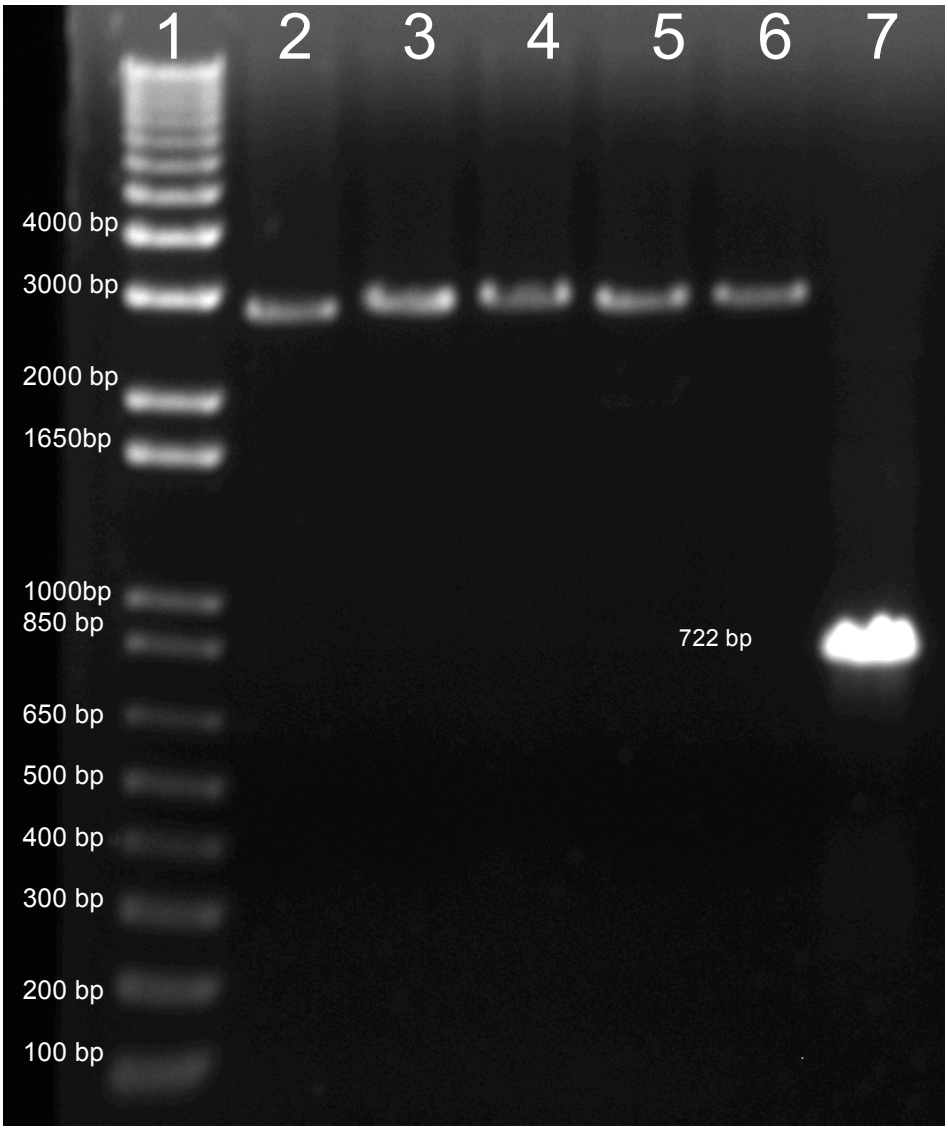

Figure 2.3: Digestion of the four expression vectors with *BamH*I and *Xba*I (lanes 2-6) and digestion of *gfp* with *Bgl*II and *Xba*I (lane 7).

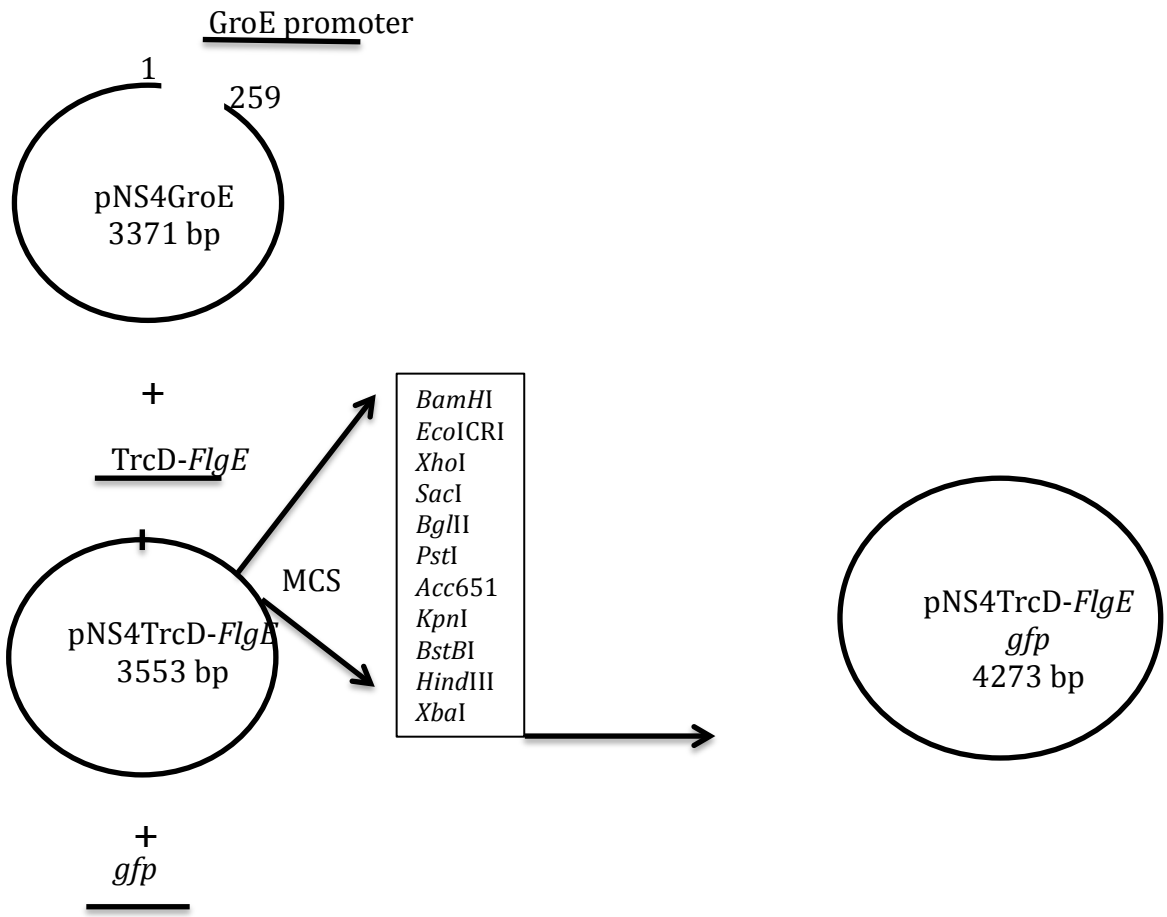

Figure 2.4: Schematic diagram depicting the cloning strategy for construction of the expression vector pNS4TrcD-*FlgE-gfp*. *groE* promoter was excised out of pNS4 expression vectors via *Sal*I and *BamH*I. The promoterless vectors was then ligated to *trcD-flgE* promoter. Gene coding for GFP was then cloned into the expression vectors using *BamH*I and *Xba*I restriction sites

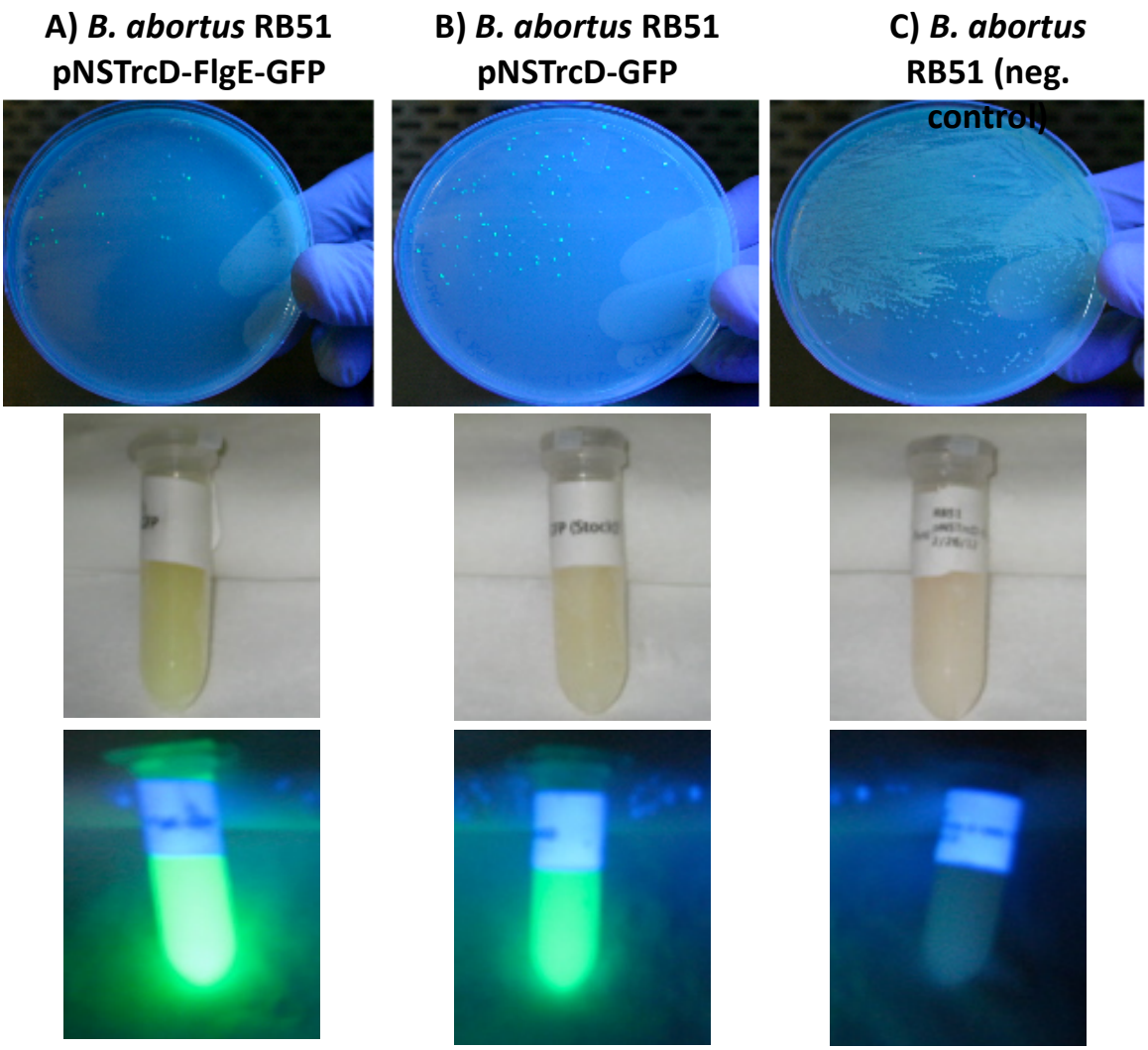

Figure 2.5: Expression of GFP in the transformed strains of RB51*leuB*$^{-}$. Expression and secretion of GFP by pNS4TrcD-FlgE (left), Cytoplasmic expression of GFP by pNS4TrcD (Middle) and non-GFP RB51*leuB*$^{-}$ (Right) under UV light.

A B

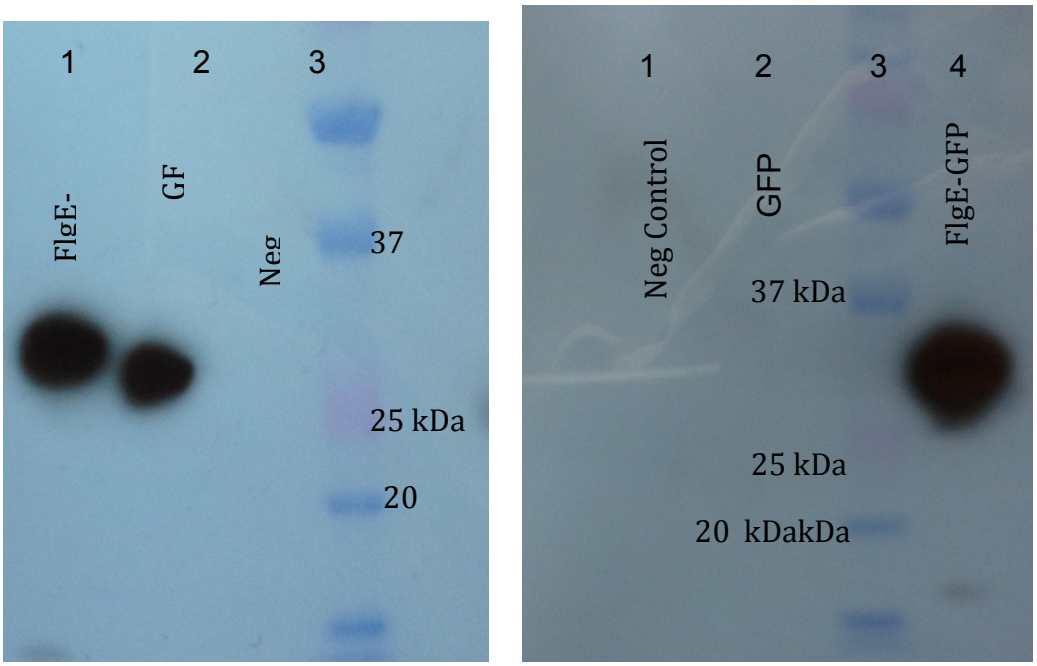

Figure 2.6: Western blotting for GFP. 6A) A crude extract from RB51*leuB*$^{-}$ cells expressing GFP along with FlgE signal sequence (lane 1), or GFP alone (lane 2). 6B) Precipitated supernatant of RB51*leuB*$^{-}$ carrying GFP constructs. GFP was found only in the supernatant when it was fused to FlgE signal sequence (lane 4). No GFP was present in the supernatant of the negative control; RB15 alone (lane 1) or RB51 expressing GFP without FlgE signal sequence (lane 2).

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Chapter 3

Expression and secretion of *M. tuberculosis* protective antigens in *B. abortus* strain RB51*leuB* and evaluation of the stability of the recombinant strains *in vitro* and *in vivo*

Abstract:

Strain RB51 is the official vaccine against cattle brucellosis in the United States and other countries. Protection conferred by strain RB51 vaccination is due to induction of cell-mediated immunity, more specifically though production of IFN-γ. In this study we have looked at the feasibility of using strain RB51^*leuB* as a vector for expression and secretion of *M. tuberculosis* antigens. Four expression vectors were designed: pNS4TrcD-Ag85B, pNS4TrcD-FlgE-Ag85B, pNS4TrcD-Rv2660c-ESAT6, and pNS4TrcD-FlgE Rv2660c-ESAT6. These expression vectors were able to express and secreted antigens Ag85B and fusion Rv2660-c-ESAT6 of *M. tuberculosis*. *In vivo* and *in vitro* studies indicated that the recombinant vaccines are stable in selective and non-selective environment. These recombinant vaccines could potentially lead to protection against brucellosis as well tuberculosis infections.

Introduction:

Brucella abortus strain RB51 is a stable rough mutant derived from the standard virulent strain *B. abortus* 2308 [1]. The efficacy of strain RB51 to induce significant protection against *B. abortus* infection in mice and cattle has been well demonstrated [2-4]. For the last two decades strain RB51 has been the official vaccine for cattle brucellosis in the United States and multiple other countries [5]. Protection afforded by strain RB51 vaccination is due to induction of cell-mediated immunity, more specifically mediated by production of IFN-γ [4, 6, 7].

Several studies have exploited the use of strain RB51 as a platform for plasmid-based expression of protective antigens of other intracellular pathogens in which cell-mediated immunity is essential for protection. Using a mouse model, these studies have concluded that immunization with recombinant strains of RB51 expressing heterologous proteins leads to a Th1 type of immune response specific to the expressed heterologous proteins without altering its vaccine efficacy in protecting against *B. abortus* challenge [6, 8, 9].

Although these findings suggest that strain RB51 could potentially be used

as a bivalent vaccine against other intracellular pathogens, additional modifications for overexpression are necessary to enhance the protective efficacy of strain RB51 as a bivalent vaccine. Such modifications should include the use of stable expression vectors and strong promoters, codon optimization of the heterologous genes, and subcellular localization of expressed antigens. These modifications would allow strain RB51 to efficiently deliver protective foreign antigens of other intracellular pathogens such as *Mycobacterium tuberculosis* [10].

We reasoned that all of the advantageous vaccinal qualities of strain RB51 could be exploited by utilizing the two expression vectors developed and modified in chapter two of this dissertation for the expression and delivery of protective antigens of *Mycobacterium tuberculosis* in which a Th1 type immune response is essential for protection.

Experimental vaccinations with mycobacterial culture filtrates (CF) have demonstrated that secreted and extracellular proteins are very antigenic. These proteins induce T-cell responses and are protective against *M. tuberculosis* challenge in several experimental models [11]. Antigen 85B (Ag85B) and the early secretory antigenic target 6 (ESAT6) are among the most dominant protective antigens present in culture filtrate of all

mycobacterial species, except in the case of BCG, which lacks ESAT6 [12]. Rv2660c is another protective antigen of *M. tuberculosis* that was recently identified, however it remains unknown if this antigen is secreted into culture filtrate.

The aim of this chapter is to utilize the constructed expression vectors; pNS4TrcD and pNS4TrcDflgE to express and secrete *M.* tuberculosis protective antigens Ag85B, ESAT6 and Rv2660c in strain RB51*leuB*. Additionally, this chapter will evaluate the stability of such recombinant strains *in vitro* using green fluorescent protein as a reporter, and in mice through recovery of the recombinant strains on leucine deficient *Brucella* minimal medium (BMM*leu*-) during the entire period of vaccine clearance.

Materials and Methods:

Recombinant DNA methods (DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis) were performed according to standard techniques [13]. The polymerase chain reaction (PCR) was performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and a Gradient Mastercycler® (Eppendorf). Oligonucleotides were purchased from Sigma-Genosys (Sigma-Aldrich, USA). Restriction and modification enzymes were purchased from Promega. QIAprep Spin Miniprep Kit from

QIAGEN was used for plasmid extractions and QIAGEN PCR cleanup kit was used for restriction enzyme removal and DNA gel extraction.

Generation of recombinant strain RB51*leuB* expressing Ag85B

The *fbpB* and *rv2660c* genes encoding Ag85B and Rv2660c antigens, respectively, were amplified via polymerase chain reaction (PCR) from the genomic DNA of *M. tuberculosis*. Forward and reverse primers (Table 3.1) were designed based on the nucleotide sequence (GenBank accession no. AFN49815.1 for *ag85B* and GenBank accession no CCP45458.1 for *rv2660c*); *BamH*I and *Xba*I restriction sites were engineered into the forward and reverse primers, respectively, to facilitate directional cloning into pNS4TrcD and pNS4TrcDFlgE expression vectors. A *Bgl*II restriction site was also designed in the reverse primers, upstream of *Xba*I restriction site, for subsequent cloning of other genes downstream *fbpB* or *rv2660c*. The amplified DNA regions (*ag85B* and *rv2660c*) (Figure 3.1) were digested with *BamH*I and *Xba*I restriction enzymes and subcloned into the same sites of pNS4TrcD and pNS4TrcDFlgE plasmids to generate pNS4TrcD–*ag85B*, pNS4TrcD–*rv2660c*, pNS4TrcDFlgE-*ag85B* and pNS4TrcDFlgE-*rv2660c* expression vectors.

Generation of recombinant strain RB51*leuB* expressing fusion Rv2660c-ESAT6

The *rv2660c-esat6* fusion was accomplished in two steps (Figure 3.2). In the first step, *rv2660c* was cloned as described earlier. In the second step, the gene coding for ESAT6 was amplified via PCR from the genomic DNA of *M. tuberculosis*. Forward and reverse primers were designed based on the nucleotide sequence of *esat6* (GenBank accession no. ABD98028.1) *BamH*I, *Xba*I and *Bgl*II sites were engineered into the primers in the same manner as the primers for *ag85B* and *rv2660c* (Table 3.1). Amplified *esat6* was then digested with *BamH*I and *Xba*I restriction enzymes and subcloned into the same sites of pNS4TrcD–rv2660c and pNS4TrcDFlgE-*rv2660c* to generate pNS4TrcD–*rv2660c-esat6* and pNS4TrcDFlgE-*rv2660c-esat6* expression vectors.

Generation of recombinant strain RB51*leuB* expressing Ag85B and fusion Rv2660c-ESAT6 from codon-optimized synthetic genes

Genes encoding antigens Ag85B, ESAT6 and Rv2660c were synthesized by Genscript (NJ, USA) using *Brucella* codons. *BamH*I, *Bgl*II and *Xba*I sites were engineered into the sequences in the same manner as the original genes. The synthetic genes were then cloned into pNS4TrcD and pNS4TrcDFlgE as

described previously.

Preparation of the protein extract and Western Blotting

All constructed vectors carrying *M. tuberculosis* protective antigens were electroporated into strain RB51*leuB* as described earlier [14]. Transformants were selected by plating on (BMM*leu*-) agar. Western Blotting was performed on each recombinant strain using anti-His antibodies to confirm expression and secretion of Ag85B and fusion Rv2660c-ESAT6. Briefly, cultures of strain RB51*leuB* carrying expression vectors, were grown in BMM*leu*- to mid-log phase. For cytoplasmic expression of Ag85B and the fusion peptide Rv2660c-ESAT6, one hundred microliters of each culture were pelleted for 5 minutes in a microcentrifuge tube at 12,000 x G. The pellet was then lysed with β-mercaptoethanol in the presence of 10 mM Tris-base and heated in a water bath at 95ºC for 10 minutes. For secreted Ag85B and Rv2660c-ESAT6 fusion, 1 mL of each culture was pelleted for 15 minutes in a microcentrifuge tube at 12,000 x G. The collected supernatant was centrifuged again to remove residual bacterial cells. Supernatants were then precipitated overnight at 4ºC in 10% trichloroacetic acid (TCA), centrifuged at high speed for 15 minutes and washed twice with

5 ml of pre-chilled acetone to remove residual TCA. Air-dried pellets were dissolved in 30 μl of SDS-PAGE sample buffer. Western Blot analysis was carried on proteins transferred onto nitrocellulose membranes and probed with 1: 4000 dilutions of mouse IgG2a anti-His, horseradish peroxidase (HRP) conjugated primary antibodies (Life Sciences, Inc., USA).

Cloning of GFP into expression vectors

Gene coding for green fluorescent protein [15]was amplified via polymerase chain reaction (PCR) using pNSTrcD-*gfp* [16] as a template and the primers listed in (Table 3.1). The PCR amplicon was gel purified, digested with *Bgl*II and *Xba*I and cloned into the four expression vectors, which were previously digested with *Bgl*II and *Xba*I, to create pNS4TrcD-*ag85b-gfp*, pNS4TrcDFlgE-*ag85B-gfp*, pNS4TrcD–*rv2660c-esat6-gfp* and pNS4TrcDFlgE-*rv2660c-esat6-gfp* expression vectors. All plasmid constructs carrying genes encoding *M. tuberculosis* antigens along with GFP were electroporated into strain RB51*leuB* as described earlier [14]. Transformants were selected by plating on BMM*leu-* agar. RB51*leuB* transformants harboring the constructed vectors along with the reporter protein, GFP, were visualized under UV light to detect green fluorescent colonies. These green appearing transformants were later screened for the presence of the expression vectors by plasmid extraction and restriction

digestion.

Evaluation of plasmid stability *in vitro*

To evaluate the stability of the newly constructed vectors expressing *M. tuberculosis* protective antigens along with GFP, three random colonies from each construct (Table 3.2, strains 6&7) were subcultured onto either non-selective-enriched medium (TSA) or selective medium (BMM*leu*-). The subcultures were evaluated for GFP expression under UV light and, subsequently, subcultured onto new plates every 4 days until fluorescence was no longer observed.

Evaluation of plasmid stability in BALB/c mice

Five groups of 4-6 week old BALB/c mice (n=9) were vaccinated with approximately 2-5 x10^{8} CFU of RB51*leuB* carrying pNS4TrcD–*rv2660c-esat6* pNS4TrcDFlgE-*rv2660c-esat6* pNS4TrcD–*ag85B*, pNS4TrcDFlgE-*ag85B* or pNS4GroE (Negative control). Three mice from each group were euthanized on weeks 3, 6 and 8 post vaccination. Spleens and livers were aseptically harvested, homogenized, serially diluted and plated on TSA (non-selective media) and BMM*leu*- (selective media) agar plates for determination of colony forming units (CFU) determination. The numbers of

CFUs determined from growth on BMM*leu-* were compared to the CFUs determined from growth on TSA to measure of plasmid stability *in vivo*. Colonies isolated on BMM at week 6 were subjected to western blotting for confirmation of protein expression i.e mycobacterial antigens (data not shown).

Results:

Construction of expression vectors:

Genes encoding *M. tuberculosis* protective antigens were successfully amplified from genomic DNA and cloned into the two expression vectors to create pNS4TrcD–*rv2660c-esat6* pNS4TrcDFlgE-*rv2660c-esat6* pNS4TrcD–*ag85B* and pNS4TrcD*flgE-ag85B* for cytoplasmic expression and secretion of *M. tuberculosis* protective antigens. However; upon transformation of the expression vectors into strain RB51*leuB*, recombination events took place within the mycobacterial genes leading to mutation and thereby prevention of expression of the intended proteins (Figure 3.3). To overcome this hurdle, genes encoding antigens Ag85B, ESAT6 and Rv2660c were synthesized using *Brucella* codons and cloned into the expression vectors. Expression and secretion of the synthesized genes were confirmed by Western Blotting as shown in (Figure 3.4).

Additionally, green fluorescent protein (GFP) reporter gene was cloned downstream of the synthesized mycobacterial genes as a chimeric protein to monitor the expression of the recombinant proteins (Figure 3.5). A linker consisting of Ala-Ala-Gly-Gly-Ser-Glu-Lys amino acid sequence was inserted between mycobacterial antigens and GFP to allow for proper folding of GFP.

Evaluation of recombinant RB51 strains stability *in vitro*

To evaluate the stability of the newly constructed vectors expressing mycobacterial proteins along with GFP as one chimeric protein, three random colonies of each construct were subcultured onto either non-selective-enriched medium (TSA) or selective medium (BMM*leu*-). Through evaluation of GFP expression under UV light, both expression vectors were maintained for more than 15 passages or 60 days on selective media (Table 3.3). However, when grown on non-selective enriched media the same recombinant strains were only stable up to 7-8 passages or 28-32 days as shown in (Table 3.4).

Evaluation of recombinant RB51 strains stability in mice

To evaluate the stability of the newly constructed vectors expressing mycobacterial antigens in strain RB51, five groups of BALB/c mice (9 mice

per group) were vaccinated with approximately 2-5 x10^8 CFU of RB51*leuB* carrying the constructed vectors listed in Table 3.2. Three mice of each group were sacrificed at weeks 3, 6 and 8 post-vaccination. All vaccinated mice cleared the vaccine within 6 to 8 weeks post-vaccination. The recombinant strain of RB51 carrying the secreted or non-secreted form of the fusion Rv2660c-ESAT6 maintained the expression vectors during the entire course of vaccine persistence, 6-8 weeks (Figures 3.6 B&C). Whereas the recombinant strain carrying the secreted and non-secreted forms of Ag85B maintained the expression vectors for 3 to 6 weeks and 6 to 8 weeks, respectively (Figure 3.6 D&E). Western Blotting of colonies isolated on week 6 showed stable expression of both Ag85B and fusion protein Rv2660c-ESAT6 post passage through mice.

Discussion:

B. abortus vaccine strain RB51 is a potent stimulator of cell-mediated immunity response. This vaccine strain acts as an immunomodulator by promoting a strong Th1 type of immune response, and simultaneously inhibiting the Th2 type of immune response. These vaccinal qualities of RB51 make it a prime candidate for the development of an RB51-based multivalent vaccine that can confer protection against brucellosis and other

intracellular pathogens that require cell-mediated immunity such as *M. tuberculosis*. In an effort to develop such multivalent vaccines, two expression vectors were created for cytoplasmic expression and secretion of recombinant proteins in strain RB51 (Chapter 2).

The experiments in this chapter test the *in vivo* and *in vitro* stability of strain RB51 in expressing and secreting mycobacterial protective antigens. To do this, the gene sequences of *M. tuberculosis* proteins, Ag85B and fusion Rv2660c-ESAT6, were cloned into pNS4TrcD and pNS4TrcDFlgE expression vectors. Expression of these recombinant proteins was not achieved using the original DNA sequences of *M. tuberculosis*. This lack of expression was due to mutations within the mycobacterial genes. Such mutations were attributed to the difference in codon usage between *Mycobacterium* species and *Brucella* species [17]. Codon Adaptation Index (CAI) [18] was used to predict the expression level of mycobacterial proteins in strain RB51. This index uses a reference set of highly expressed proteins from a bacterial species to assess the relative merits of each codon, and a score for the gene, ranging from zero to one, is calculated from the frequency of use of all codons in that gene. Using CAI, it was shown that the predicted levels of expression of Ag85B, Rv2660c and ESAT6, using original DNA sequences of *M. tuberculosis*, were 0.51, 0.29 and 0.47,

respectively. It has been well documented that the rate of translation of foreign antigens in wild-type vectors can be influenced by their codon usage [19]. *Brucella* species have a G+C content of 57%, whereas the genomes of *Mycobacterium* species have a higher G + C content of 65.9%, [20, 21]. As a result, there is a high degree of bias for codons with G and C at the third nucleotide position, effectively, leading to codons that are rare in *Brucella* species. This use of rare codons usually compromises expression of heterologous proteins by inducing translational errors such as stalling, termination, amino acid substitution and possibly frameshifting [17]

One of the approaches normally used to overcome this problem is codon optimization for genes of interest. This technology is very cost-effective and has turnaround times of only a few weeks. In our studies, genes coding for the intended mycobacterial genes were synthesized commercially using *Brucella* codons through Genscript. Upon optimizing these codons for *Brucella* expression, the predicted levels of expression of Ag85B, Rv2660c and ESAT6 became 0.94, 0.94 and 0.93, respectively (Table 3.5). This optimization of codon usage resulted in stable expression vectors and successful expression of *M. tuberculosis* proteins in strain RB51 without showing any signs of lethality or other forms of protein toxicity.

Unlike mutant strains of bacteria that are engineered for protein synthesis i.e *E. coli*, expression of heterologous antigens within wild-type strains, such as strain RB51, can necessarily impose a metabolic burden. A portion of the host bacterium's energy and materials are required to maintain the foreign DNA and express it as protein as well as maintain it without imposing toxicity on its self. In the case of recombinant vaccines, it is very crucial for such strains to maintain plasmid and expression of foreign antigens in order to develop a strong immune response against the targeted antigens. One of the techniques used to assess the stability of recombinant strains *in vivo* and *in vitro* is through estimating the percentage of recombinant colonies retaining selective markers, which is used as an indicator of retention of the plasmid. However, this approach has limitations; for example, retention of selection markers does not distinguish between retention of intact vector or retention of deletion mutants which have lost the foreign gene but retained the selection marker. Therefore a more robust confirmation is necessary not only to test for the presence of the intact expression vectors along with the foreign DNA, but also for expression of such foreign antigens. In our study GFP was used as a reporter protein to evaluate the stability of expression vectors and expression of mycobacterial antigens *in vitro*. This was done by fusing GFP in-frame downstream of the mycobacterial genes. The ATG start

codon of GFP was deleted and replaced by a linker to allow for in-frame translation and flexibility for proper GFP folding. Using this reporter system, it was shown that the recombinant strains were very stable when grown under selective pressure i.e., BMM*leu*-. However, when grown using non-selective media, the recombinant strains were stable up to 7-8 passages. The stability of the constructed expression vectors was also assessed *in vivo* by vaccinating mice with each construct. It was apparent that recombinant strains of RB51 were stable during the entire course of vaccination. An immunoblot on isolated colonies at week 6 showed stable expression of the mycobacterial antigens. These data strongly suggest that the RB51 could potentially be used as vector for the delivery of protective antigens of *Mycobacterium* species. The new RB51 vaccine strains carrying mycobacterial antigens developed in this study could potentially serve as an environmentally safe bivalent vaccine for protection against *Brucella* and *Mycobacterium* infections.

Primer ID	Primer Sequence
ag85B-F	GGGGGATCCTTCTCCCGGCCGGG
ag85B-R	GGGTCTAGATCAAGATCTGCCGGCGCCTAACG
esat6-F	GGGGGATCCATGACAGAGCAG
esat6-R	GGGTCTAGACTAAGATCTTGCGAACATCCC
rv2660c-F	GGGGGATCCGTTGGACACCAAA
rv2660c-R	GGGTCTAGACTAAGATCTGTGAAACTGGTT
ag85B-F Synthetic	GGGGGATCCTTCTCCCGCCCGGG
ag85B-R Synthetic	GGGTCTAGATTAAGATCTAGCCCGCGCCGAG
esat6-F Synthetic	GGGGGATCCATGACGGAACAG
esat6-R Synthetic	GGGTCTAGATTAAGATCTCGCGAACATGCC
rv2660c-F Synthetic	GGGGGATCCGGATCCGTTATC
rv2660c-R Synthetic	GGGTCTAGATCAAGATCTATGGAACTGAT
gfp-F with linker	GGGAGATCTGCCGCCGGCGGCAGCCAGAAGATGAGT AAAGGAGAAGAAC
Gfp-R	GG**TCTAGA**TTATTTGTAGAGCTCAT

Table 3.1: PCR primers used to amplify DNA sequences for subsequent cloning into expression vectors.

Strain Number	Strain Designation	Expression Vector	Vector Size	Antigen Expressed
1	RB51	pNS4GroE	3371 bp	------
2	RB51-Ag85B	pNS4TrcD-*ag85B*	4301 bp	Ag85B
3	RB51FlgE-Ag85B	pNS4TrcD*flgE*-*ag85B*	4481 bp	Secreted Ag85B
4	RB51-Rv2660c-Esat6	pNS4TrcD-*rv2660c-esat6*	3986 bp	Rv2660c-Esat6 fusion
5	RB51FlgE-Rv2660c-Esat6	pNS4TrcD*flgE*-*rv2660c-esat6*	4166 bp	Secreted Rv2660c-Esat6 fusion
6	RB51-Ag85B-GFP	pNS4TrcD-*ag85B-gfp*	5042 bp	Ag85B-GFP chimeric
7	RB51-Rv2660c-Esat6-GFP	pNS4TrcD-*rv2660c-esat6-gfp*	4727 bp	Rv2660c-Esat6-GFP chimeric

Table 3.2: List of expression vectors created.

Strain Designation	Number of Passages	Number of Days
RB51-Ag85B-GFP	> 15	>60
RB51-Rv2660c-Esat6-GFP	>15	> 60

Table 3.3: Stability of RB51 recombinant strains carrying *M. tuberculosis* antigens along with GFP selective media, BMM*leu*- agar.

Strain Designation	Number of Passages	Number of Days
RB51-Ag85B-GFP	7	28
RB51-Rv2660c-Esat6-GFP	8	32

Table 3.4: Stability of RB51 recombinant strains carrying *M. tuberculosis* antigens along with GFP on non-selective media, TSA.

Gene	CAI- Original DNA Sequence	CAI- Optimized DNA Sequence
Esat6	.47	.94
ag85B	.51	.94
rv2660C	.29	.93

Table 3.5: Codon Adaptation Index (CAI) for *M. tuberculosis* protein expression in strain RB51 before and after codon optimization.

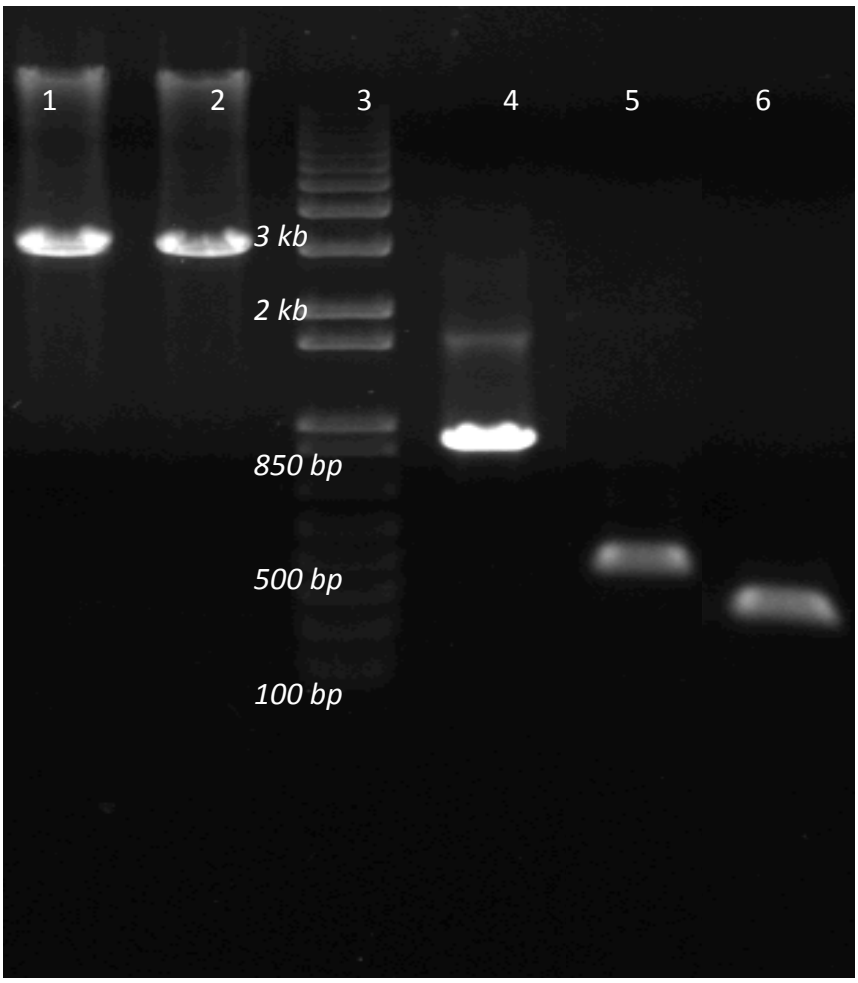


Figure 3.1: Digestion of pNS4TrcD (lane 1), pNS4TrcDFlgE (lane 2), *ag85B* (lane 4), *esat6* (lane 5) and *rv2660c* (lane 6) with *BamH*I and *Xba*I.

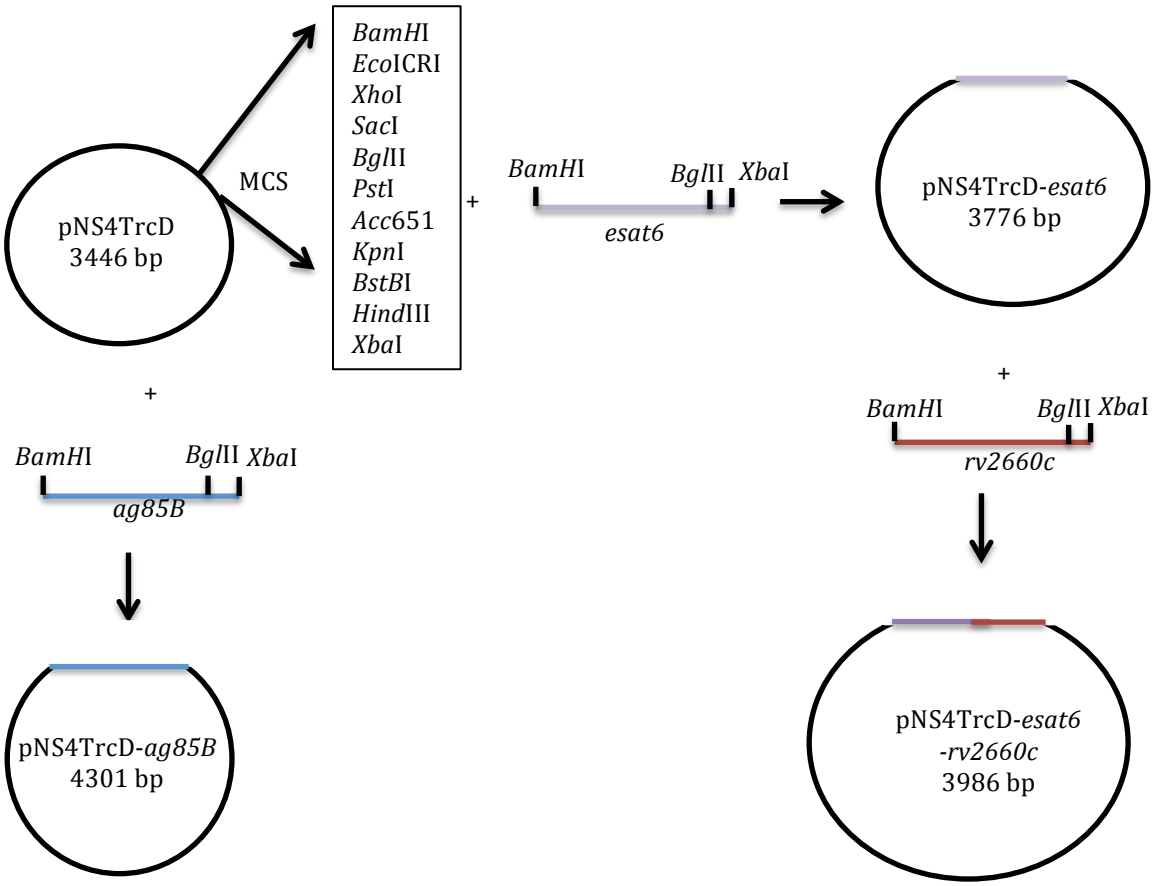


Figure 3.2: Schematic diagram depicting the cloning strategy for construction of the expression vectors carrying mycobacterial genes. *ag85B* was cloned into pNS4TrcD via *BamH*I and *Xba*I restriction sites. *rv2660c was cloned into pNS4TrcD via BamH*I and *Xba*I restriction sites. esat6 was cloned into pNS4TrcD-*rv2660c* via *Bgl*II and *Xba*I.

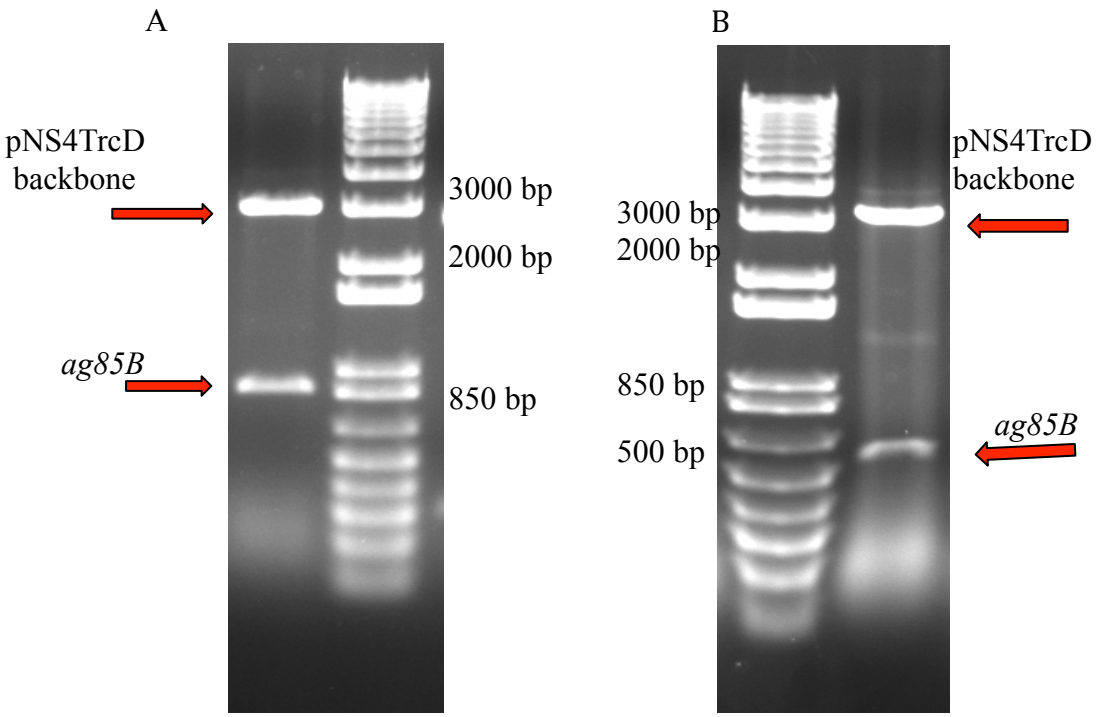
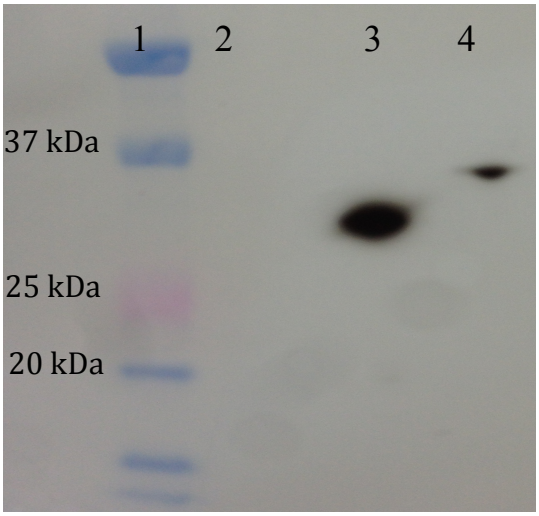
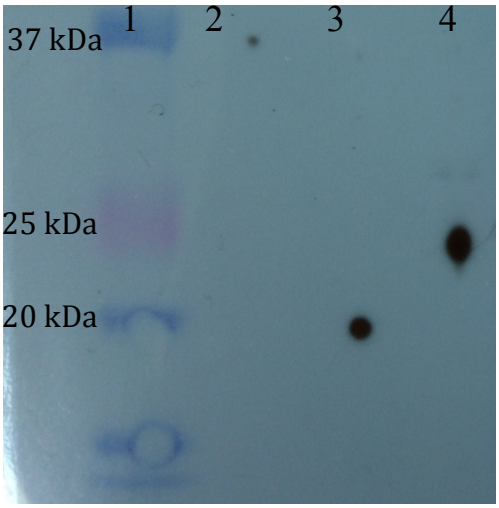


Figure 3.3: Frameshift mutation within expression vector pNS4TrcD-*ag85B* in strain RB51. A- Digestion of the expression vector by *BamH*I and *Xba*I prior to transformation into strain RB51. B- Digestion of the expression vector by *BamH*I and *Xba*I post transformation into strain RB51. The deletion occurred within the *ag85B* segment.



1- Protein ladder
2- RB51 (Negative control)
3- Ag85B (31 kDa)
4- FlgE-Ag85B (36 kDa)



1- Protein ladder
2- RB51 (Negative control)
3- Rv2660c-ESAT6 (20 kDa)
4- FlgE-Rv2660c-ESAT6 (25 kDa)

Figure 3.4: Expression and secretion of *M. tuberculosis* protective antigens in strain RB51. Top figure: Expression of Ag85B (lane 3) and the secreted form of Ag85B (lane 4). Bottom: Expression of Rv2660c-ESAT6 fusion (lane 3) and the secreted form of Rv2660c-ESAT6 (lane 4).

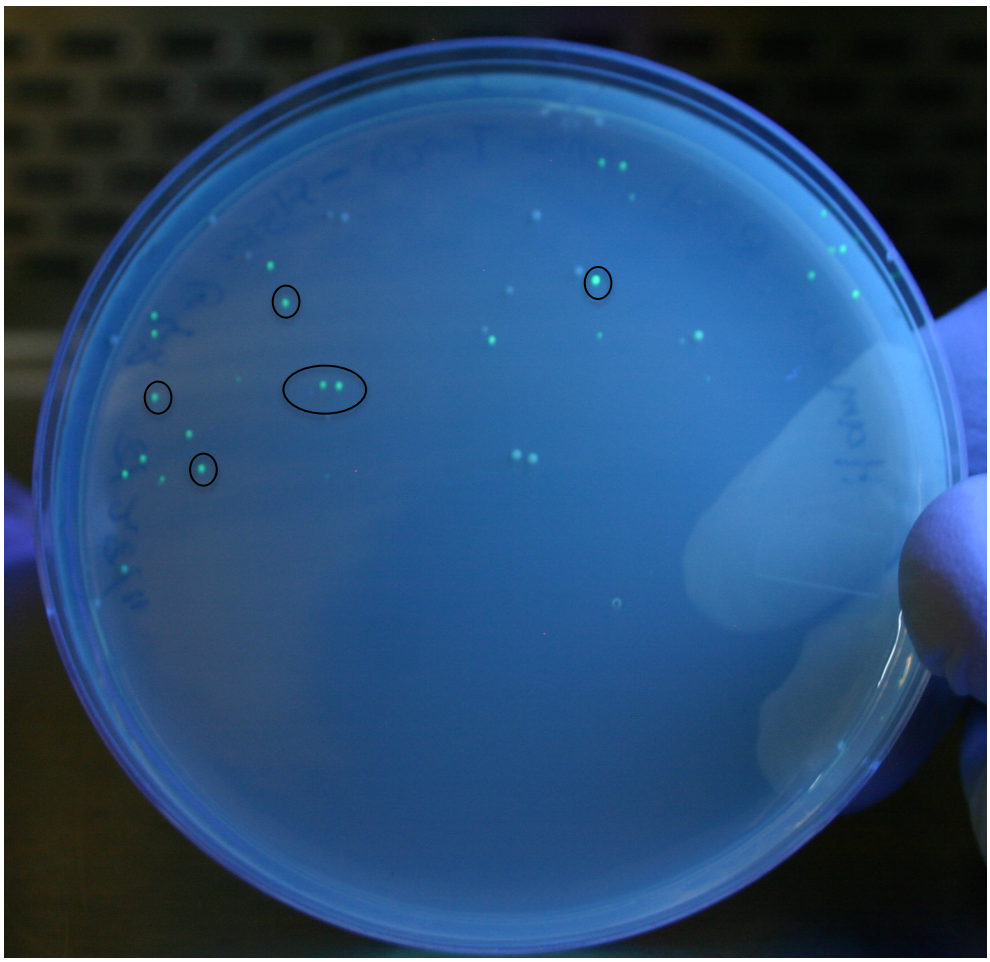


Figure 3.5: Expression of Ag85B-GFP chimeric protein in strain RB51. Picture was taken under UV light. Circled colonies indicate expression of Ag85B-GFP fusion protein.

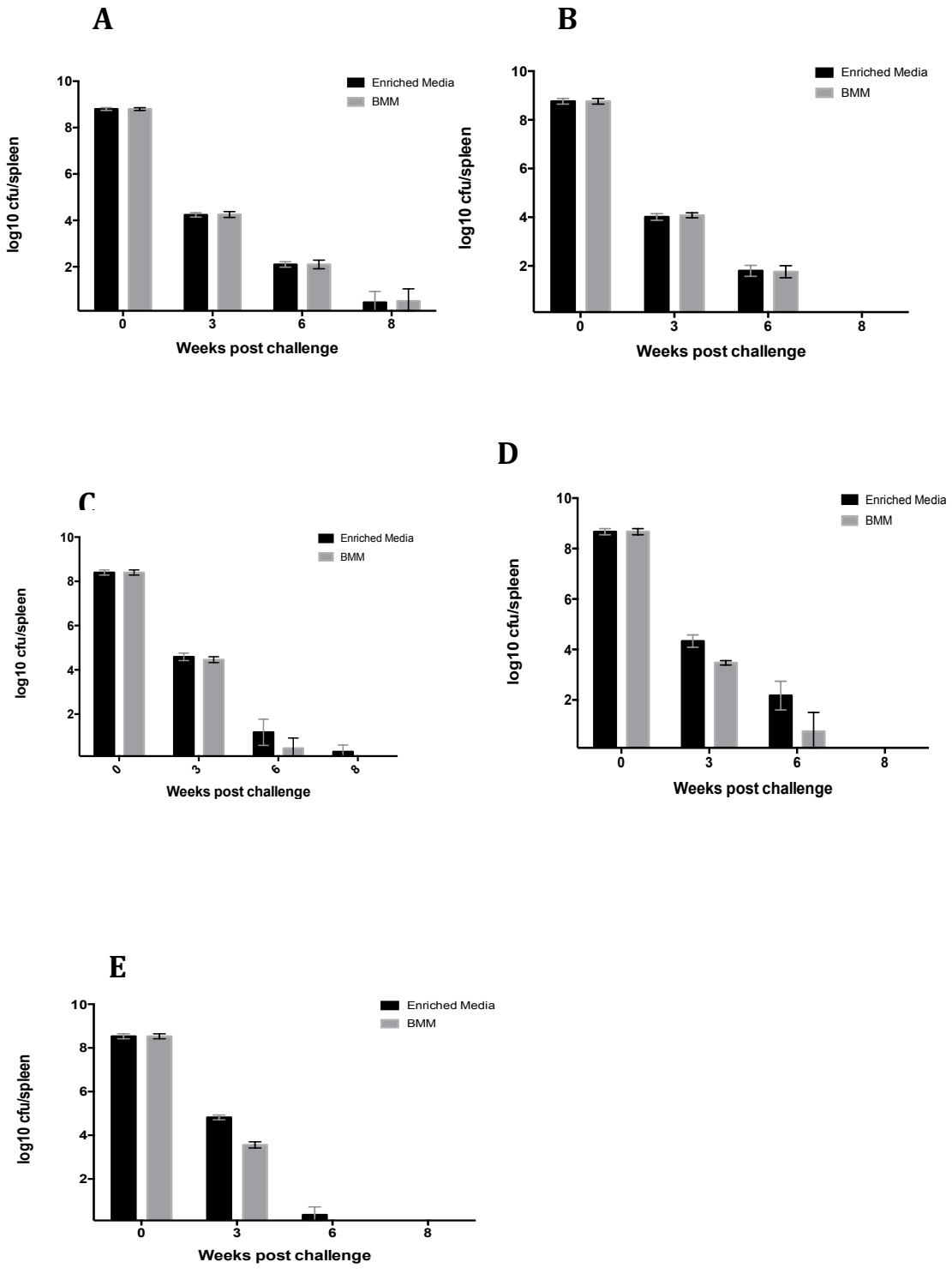


Figure 3.6: Stability of recombinant strains in mice. A) RB51 GroE (control); B) RB51-RV2660c-ESAT6; C) RB51FlgE-RV2660c-ESAT6; D) RB51-Ag85B; E- RB51FlgE-Ag85B.

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Chapter 4

The efficacy of recombinant RB51*leuB* strains against *M. tuberculosis* challenge in a mouse model

Abstract

Tuberculosis is one of the most prevalent infection diseases afflicting humankind. According to the WHO, 8. 6 million new cases of TB are reported worldwide every year. Therefore, there is a need for a vaccine that would prevent the initial establishment of TB infection and, subsequently, prevent transmission of the disease between healthy individuals. Here we report the efficacy of the Brucella abortus vaccine strain RB51 carrying *M. tuberculosis* protective antigens in a mouse model. Immunization with RB51 strains carrying *M. tuberculosis* protective antigens resulted in a Th1 type of immune response characterized by antigen specific induction of IgG2a and IFN-γ. Mice immunized with a combination of two strains of RB51 in equal numbers; one carrying Rv2660c-ESAT6 and another carrying Ag85B, resulted in 0.9 log protection against subsequence challenge with *M. tuberculosis*. However upon priming mice with these recombinant strains

and boosting them with subunit vaccines (Ag85B & ESAT6), significant protection (1.0 log reduction) was achieved. Additionally, this vaccine strategy led to protection similar to that conferred by BCG. These recombinant RB51 vaccine strains could potentially be used to protect against *M. tuberculosis* infection.

Introduction:

Tuberculosis (TB), caused by *M. tuberculosis,* is one of the most prevalent infectious diseases afflicting humankind. The World Health Organization (WHO) estimates that one third of the world's population, approximately 2.2 billion people, are infected with TB with a mortality of 1.7 million people annually [1, 2]. In the 2012 TB report by the WHO, there were approximately 8.6 million new cases of human TB globally reported. Tuberculosis is also a leading cause of death among Human Immunodeficiency Virus (HIV) infected people, with co-infection accounting for up to 29% of deaths [3].

Currently, the only licensed vaccine approved against tuberculosis is the attenuated *M. bovis* Bacille Calmette-Guérin, BCG. This live attenuated vaccine has been in use for almost a century and has displayed protective efficacy against serious forms of the disease, e.g., meningitis, in children.

However, the efficacy of BCG against pulmonary TB in adults has been variable and inconsistent (0-80%) [2, 4]. The lack of an effective vaccine against TB along with the emergence of multidrug-resistant (MDR) and the more recent occurrence of totally drug-resistant (TDR) strains of *M. tuberculosis* warrant an improved second-generation vaccine that can act as an efficient prophylactic vaccine against TB.

Due to the complexity of the host immune response against TB infection and the genetic restriction imposed by major histocompatibility complex molecules, it has become evident that vaccines containing multiple epitopes are more effective than single-peptide vaccines [27]. Such effective vaccines must be able to elicit and activate the cellular arm of the immune system (CMI) in order to induce protection against TB. Numerous mechanisms have been used to enhance CMI. These include the use of adjuvants, DNA vaccines and human live vectors like Typhoid *Vaccine Live* Ty21a and BCG [9, 19, 27]. However, little to no attention has been focused on other live vectors such as the *B. abortus* vaccine strain RB51, due to safety concerns.

The feasibility of expressing foreign proteins in strain RB51 and its ability to induce CMI specific to the expressed foreign proteins without altering its vaccine efficacy against wild-type *B. abortus* 2308 make strain RB51 a

prime candidate for the delivery of *M. tuberculosis* antigens. Although several other modifications would be needed before it may be introduced as a human vaccine, testing the potential of RB51 as a platform for expression of mycobacterial antigens and protection against *M. tuberculosis* experimental challenge is warranted.

Secreted proteins of *M. tuberculosis* have been shown to be among the most immunogenic antigen species yet identified. These antigens include the Ag85B (35 kDa) and the early secretory antigenic target, also known as ESAT6 (10 kDa) [5-7]. Additionally, proteins associated with the latent from of TB, such as Rv2660c (9 kDa), have also been shown to be immunogenic [8]. Combining these three antigens in a subunit vaccine has provided significant levels of protection similar to that of BCG [9].

In chapter 3, the use of the attenuated vaccine strain RB51 as a delivery platform for *M. tuberculosis* antigens was described. Constructed recombinant strains of RB51 were shown to express and secrete *M. tuberculosis* protective antigens: Ag85B, ESAT6, and Rv2660c. Furthermore, these recombinant strains were shown to be very stable *in vitro* and *in vivo*. The purpose of the present study is to evaluate the protective efficacy of these recombinant strains of RB51 in providing protective

immunity against *B. abortus* as well as *M. tuberculosis* challenge in mice.

Materials and Methods:

Animals

Female BALB/c mice (6-8 weeks old, Harlan Laboratories, Indianapolis, IN) were used in all experiments to evaluate the immune response to the recombinant RB51 vaccine strains. All the animal experimental protocols were approved by Institutional Animal Care and Use Committee (protocol # CVM-13-070) at Virginia Tech and carried out in our AAALAC approved and CDC approved ABSL3 facility. For retro-orbital bleeding, mice were anaesthetized under isoflurane using a Vet Equip Mobile Laboratory Animal Anesthesia System. Mice were euthanized using overdose of carbon dioxide in-cage followed by cervical dislocation [10].

Preparation of bacterial strains

For preparation of the vaccine constructs and *B. abortus* 2308 challenge dose, four plates of leucine deficient *Brucella* minimal medium (BMM*leu*-) and TSA (for *B. abortus* 2308) were seeded with each strain and incubated for four days at 37ºC in 5% CO_2. Lawns of bacteria were scraped off the agar with a sterile loop and suspended in 20 ml of Phosphate Buffer Saline, PBS. The suspensions were centrifuged at 12,000 x G for 30 minutes at 4ºC,

washed twice with PBS (25 ml), resuspended in 5 ml of 15% glycerol, aliquoted and stored at -80 C until use. After one week of storage, the actual colony forming unit (CFU) was determined and recorded.

For preparation of *M. tuberculosis* H37Rv (ATCC 25618) challenge dose, 25 ml of 7H9 Middlebrook Mycobacteria base supplemented with ADC Enrichment Medium (Sigma-Aldrich, USA) were inoculated with *M. tuberculosis*. Culture was grown in a shaking incubator at 37ºC up to 100 Klett units mid-log phase). The strains were recovered by centrifuging each culture at 12,000 x G for 30 minutes at 4 ºC. Strains were then washed twice with PBS (25 ml), resuspended in 5 ml of 15% glycerol, aliquoted and stored at -80 C until use. Actual CFU count was determined after one week of storage as described above using 7H10 agar plates.

Mouse Experiment 1: Enzyme-Linked Immunoabsorbent Assay (ELISA)

A total of 15 BALB/c mice were divided into 5 experimental groups (n=3). Group 1 was immunized with strain RB51 (control). Group 2 was immunized with strain RB51 carrying Ag85B. Group 3 was immunized with strain RB51 carrying FlgE-Ag85B. Group 4 was immunized with strain RB51 carrying Rv2660c-ESAT6. Group 5 was immunized with strain RB51 carrying FlgE-Rv2660c-ESAT6. Blood was collected from the different

groups of mice by retro-orbital bleeding at weeks 3 and 6 post-vaccination. Levels of immunoglobulin (total IgG) as well as IgG1 and IgG2a, with specificity to Ag85B and ESAT6, in serum samples were determined by indirect ELISA as described before [60, 65]. Briefly, antigens Ag85B and ESAT6 (Provided by BEI Resources, VA, USA) were diluted in carbonate buffer, pH 9.6, at a concentration of 5 μg/ml. Wells of polystyrene plates (Nunc-Immunoplate with maxisorp surface) were coated with each antigen at 0.5 μg/well. Following overnight incubation at 4°C, plates were washed four times with washing buffer (PBS at pH 7.4, 0.05% Tween 20) and blocked with 2% bovine serum albumin (BSA) in PBS. After 1-hour incubation, plates were washed 4 times with 200 ul of washing buffer and incubated with the appropriate mouse sera diluted in 100 μl blocking buffer. The plate were incubated for 4 hours at room temperature and washed four times with 200 ul of washing buffer. Horseradish peroxidase-labeled goat anti-mouse isotype specific conjugates (Southern Biotechnology, AL-USA) were added to the wells at 1:4000 dilution. After 1 hour of incubation at room temperature, the plates were washed four times with 200 ul of washing buffer. One hundred μl of substrate solution (TMB Microwell peroxidase substrate, (KPL, MD, USA) were applied to each well. After 15-20 minutes of incubation in the dark at room temperature, the enzymatic reaction was

stopped by adding 100 µl of stop solution (0.185 M sulfuric acid) and absorbance was measured at 450 nm using a microplate reader.

Mouse experiment 2: Splenocyte proliferation assay cytokine production

A total of 15 BALB/c mice were divided into 5 experimental groups (n=3). Group 1 was vaccinated with 100 µl PBS (control). Group 2 was vaccinated with RB51 (control). Group 3 was vaccinated with *M. bovis* BCG. Group 4 was vaccinated with two strains of RB51 in equal numbers: one carrying Ag85B and another carrying Rv2660c-ESAT6 (non-secreted). Group 5 was vaccinated with two strains of RB51 in equal numbers; one carrying FlgE-Ag85B and another carrying FlgE-Rv2660c-ESAT6 (secreted antigens). The inoculation titer for the BCG group was $1x10^6$ CFUs, whereas in all RB51 vaccinated groups the titer was $2\text{-}4x10^8$ CFUs. Six weeks post vaccination mice were euthanized and spleens were collected for splenocyte proliferation assay as described before [66]. Briefly, splenocytes were collected and seeded in 96 well cell culture plates at a concentration of $1x10^5$/well. The cells (3 wells each) were stimulated with either growth medium, ConA (0.1, 1, 5 and 10 µg/well), Ag85B (0.1, 1, 5 and 10 µg/well) or ESAT6 (0.1, 1, 5 and 10 µg/well), heat killed RB51 (0.1, 1, 5 and 10 µg/well), or heat killed *M. tuberculosis* (0.1, 1, 5 and 10 µg/well). The cells were incubated at

37°C and 5% CO_2 for 5 days. Supernatants were collected from the wells and specific cytokine levels of IFN-γ, TNF-α, IL-2, IL-4 and IL-5 were determined using Bio-RAD Bio-Plex ProMouse cytokine Th1/Th2 Assay according to the manufacturer's instructions.

Mouse experiment 3: Protection against wild-type *B. abortus* 2308

Forty-five BALB/c mice were divided into 9 experimental groups (n=5). Group 1 was immunized with 100 µl PBS (control). Group 2 was immunized with RB51 (control). Group 3 was immunized with *M. bovis* BCG strain. Group 4 was immunized with RB51 carrying Ag85B. Group 5 was immunized with RB51 carrying FlgE-Ag85. Group 6 was immunized with RB51 carrying Rv2660c-ESAT6. Group 7 was immunized with RB51 carrying FlgE-Rv2660c-ESAT6. Group 8 was immunized with a combination of two strains of RB51 in equal numbers; one carrying Rv2660c-ESAT6 and another carrying Ag85B (non-secreted antigens). Group 9 was immunized with a combination of two strains of RB51 in equal numbers; one carrying FlgE-Rv2660c-ESAT6 and another carrying FlgE-Ag85B (secreted antigens). The inoculation titer for the BCG group was $1x10^6$ CFU, whereas in all RB51 vaccinated groups the titer was $2\text{-}4x10^8$ CFU. Six weeks post vaccination, all nine groups were challenged with

$5x10^4$ CFU of *B. abortus* 2308 (i.p). Two weeks post challenge all mice were sacrificed. Spleens and livers were removed aseptically, homogenized, serially diluted, plated on TSA agar, and incubated for four days at 37ºC in 5% CO_2.

Mouse Experiment 4: Protection against *M. tuberculosis* challenge

Forty five BALB/ c mice were divided into 9 experimental groups (n=5). All groups were vaccinated with same strains and doses as those in the *B. abortus* challenge study. Eight weeks post vaccination, all nine groups were challenged with 2x10^5 CFU of *M. tuberculosis* (i.p). Four weeks post-challenge all mice were sacrificed. Spleens and lungs were removed aseptically, homogenized, serially diluted and plated on 7H10 selective media and incubated for 2-3 weeks at 37ºC in 5% CO_2.

Mouse experiment 5: Protection against *M. tuberculosis* challenge following priming with RB51 recombinant strains and boosting with subunit vaccine

Twenty BALB/ c mice were divided into 4 experimental groups (n=5). Groups 1 & 2 were immunized with 100 µl PBS (control). Group 3 was immunized with *M. bovis* BCG strain. Group 4 with two strains of RB51 in equal numbers; one carrying Rv2660c-ESAT6 and another carrying Ag85B

(non-secreted antigens). Six weeks post-vaccination; groups 2 & 4 were boosted with 20 μg of Ag85B and 20 μg of ESAT6 in DDA (250 μg /dose; Avanti Polar Lipids, Inc.) with 25 μg of MPL (Avanti Polar Lipids, Inc.) in a volume of 100 μl subcutaneously. Blood was collected from the different groups of mice by retro-orbital bleeding one week pre-boosting and one week post-boosting. Levels of immunoglobulin (total IgG) as well as IgG1 and IgG2a, with specificity to Ag85B and ESAT6, in serum samples were determined by indirect ELISA as described earlier. Two weeks post-boosting, all groups were challenged with $2x10^5$ CFU of *M. tuberculosis* (i.p.). Four weeks post challenge all mice were sacrificed. Spleens and lungs were removed aseptically, homogenized, serially diluted and plated on 7H10 selective media and incubated for 2-3 weeks at 37ºC in 5% CO_2.

Statistical Analysis

Analysis of variance (ANOVA) was used to evaluate the differences in the protection and the production of cytokines and antibodies amongst the experimental and control groups of mice. Comparisons between two groups were performed with the Student's T-test. Unless otherwise stated the level of statistical significance was set at 0.05.

Results:

Generation of specific immune responses

In the first experiment, serum was collected from mice vaccinated at weeks three and six, and tested for the presence of serum immunoglobulin G and its isotypes (IgG, IgG1, and IgG2a) with specificity to Ag85B and ESAT6 via indirect ELISA. As expected, mice vaccinated with strain RB51 carrying Ag85B or secreted Ag85B, but not those vaccinated with strain RB51 or saline, developed Ag85B-specific IgG (Figure 4.1A). Upon analyzing the subisotype of IgG detected, it was shown to be predominantly IgG2a and not IgG1 (Figure 4.1B). Similar but lower levels of IgG and its subisotype IgG2a were also detected in mice vaccinated with strain RB51 carrying Rv2660c-ESAT6 and the secreted Rv2660c-ESAT6, but not in mice vaccinated with strain RB51 or saline (Figure 4.2). Additionally, similar but higher levels of RB51-specific IgG and its serotype IgG2a were observed in all mice vaccinated with the recombinant strains of RB51 as well as the parent strain but not in mice vaccinated with saline (Figure 4.3).

In the second immunology experiment, total splenocytes were isolated from vaccinated mice six weeks post-vaccination and stimulated with heat-killed *M. tuberculosis*, heat-killed *B. abortus* RB51 six weeks post-vaccination. As expected, significant levels of interferon gamma (IFN-γ) were detected in

mice vaccinated with RB51 strains and BCG when stimulated with heat killed heat killed *B. abortus* RB51 and *M. tuberculosis*, respectively (Figure 4.4). Similar observations, in regards to antigen specific stimulation of tumor necrosis factor-alpha (TNF-α), were made when the same groups of mice were stimulated with the same antigens, however; higher levels of TNF-α were produced when splenocytes were stimulated with heat-killed *M. tuberculosis*, be it RB51 or BCG vaccinated groups (Figure 4.5).

When the same splenocytes were stimulated with Ag85B, higher levels of antigen specific IFN-γ and TNF-α were produced in mice vaccinated with BCG, RB51-Ag85B/ESAT6 and RB51FlgE-Ag85B/ESAT6 than in mice vaccinated with RB51 alone (Figures 4.4 & 4.5). However, when the same splenocytes were stimulated with ESAT6, no increase in antigen specific IFN-γ and TNF-α was observed between any of the groups (Figures 4.4 & 4.5). No levels of IL-2, Il-4, or IL-5 were detected in the culture supernatants of splenocytes stimulated with any of the antigens (data not shown).

B. abortus 2308 protection study

Based on colony forming units (CFU) count from homogenized spleens, all recombinant strains of RB51 carrying *Mycobacterial* antigens (except for RB51-Ag85B) were able to provide significant levels of protection against subsequent challenge with *B. abortus* 2308 (1-2 log reduction) compared with the unvaccinated control group. Mice vaccinated with RB51-Ag85B did provide protection (1.3 log reduction), however; it was not significant ($p = .09$). *M. bovis* BCG vaccinated group did not provide any protection against *B. abortus* 2308 challenge (Figure 4.6A). Similar protection levels were observed in all groups of mice when assessing CFU in the livers (Figure 4.7A). Mice vaccinated with a combination of RB51 strains carrying both Ag85B and fusion Rv2660c-ESAT6 in the secreted or non-secreted form also provided significant levels of protection similar to that of the parent strain (Figures 4.6B and 4.7B).

M. tuberculosis protection study

Based on CFU count from homogenized spleens, only the BCG vaccinated group resulted in significant protection (1.9 log reduction). Individual strains of RB51 carrying the secreted or non-secreted forms of Ag85B or the fusion Rv2660c-ESAT6 did not result in any level of significant protection

compared to non-vaccinated group. Mice vaccinated with two strains of RB51 carrying Ag85B and fusion Rv2660c-ESAT6 (Group 8) led to a 0.9 log reduction in CFU burden; however, significance nearly reached borderline ($p = 0.052$). Whereas, mice vaccinated with two strains of RB51 carrying the secreted forms of the same antigens did not provide protection (Figure 4.8).

A similar outcome in the lungs was observed in all groups of mice based on CFU count. Only the BCG vaccinated group resulted in significant protection (1.1 log reduction). None of the individual groups resulted in significant level of protection. Mice vaccinated with two strains of RB51 carrying Ag85B and fusion Rv2660c-ESAT6 (Group 8) led to a 0.8 log reduction in CFU burden; however, the level of protection was outside the conventional levels of significance ($p=0.13$) (Figure 4.9). No similar observations were seen in mice vaccinated with two strains of RB51 carrying secreted forms of Ag85B and fusion Rv2660-Esat6.

M. tuberculosis protection study post-boosting with subunit vaccine

Serum samples from vaccinated mice were collected one week before boosting with Ag85B and ESAT6 and one week after boosting to test for the presence of immunoglobulin G and its isotypes (IgG1, and IgG2a) with

specificity to Ag85B and ESAT6 via indirect ELISA. Increased levels of antigens specific IgG and IgG2a were observed in mice boosted with subunit vaccines. However, the levels of the antibodies detected after boosting was higher in mice primed with RB51 carrying Ag85B and RV2660c-ESAT6 than with mice inoculated with PBS alone (Figures 4.10 & 4.11).

Based on CFU count from homogenized spleens, mice vaccinated with two strains of RB51 carrying Ag85B and Rv2660c-ESAT6 and boosted with proteins Ag85B and ESAT6 resulted in significant level of protection (1 log reduction) compared to the PBS vaccinated group. Mice vaccinated with BCG alone also resulted in similar level of protection (1 log), whereas mice vaccinated with Ag85B and ESAT6 alone resulted in 0.5 log reduction than PBS vaccinated group. All protection levels were significant (Figure 4.12A).

Based on CFU count from homogenized lungs, all three vaccinated groups: Ag85B/ESAT6, BCG and RB51Ag85B/Rv2660c-ESAT6 resulted in significant protection: 0.7, 2.25 and 1.75 log reduction, respectively, compared to PBS vaccinated mice (Figure 4.12B). All protection levels conferred by each vaccinated groups was significant compared to the control group. However, the protection levels amongst the vaccinated groups were not significant.

Discussion:

Previously we have constructed recombinant strains of the *B. abortus* vaccine RB51 capable of expressing and secreting mycobacterial protective antigens Ag85B, Rv2660c and ESAT6. In an attempt to test the protective efficacy of these recombinant vaccines, a series of animal experiments were conducted. BALB/c mouse was the model of choice as this model is one the best rodent models that can be used to evaluate both brucellosis and tuberculosis infections [11, 12]. Since *Brucella* protection studies are based on intraperitoneal vaccination followed by intraperitoneal challenge with the virulent strains, we decided to follow the same route of vaccination and challenge for the *M. tuberculosis* protection study. Although this is not the natural route of tuberculosis infection, this route results in chronic infection in mouse spleens and lungs similar to that observed during low dose aerosol infection [13]. Additionally, this route of challenge ensures that a proper dose of the bacterium is given and also results in low levels of cross contamination between animals [13].

Using BALB/c mouse model, we have presented a novel vaccine strategy designed to confer protection similar to the official tuberculosis vaccine, BCG. We hypothesized that the *B. abortus* vaccine RB51 carrying

mycobacterial protective antigens could lead to protection against *M. tuberculosis* challenge in a murine model. Strain RB51 was chosen based on its ability to stimulate cell-mediated immunity not only against *B. abortus* challenge, but also against other heterologous proteins expressed by the strain [14-18]. However, these vaccinal qualities of strain RB51 had to be optimized for better induction of immune response. Our initial hypothesis was that modifying the mode of antigen delivery from cytoplasmic expression to secretion would result in a better protection level. This hypothesis was based on the fact that the *M. tuberculosis* antigens expressed in strain RB51, Ag85B and ESAT6, are naturally secreted by *M. tuberculosis* [19, 20]; therefore secreting such antigens using strain RB51 would provide a similar mode of antigen delivery. Additionally, we hypothesized that excretion of a large amount of mycobacterial antigens may prevent cytotoxicity to strain RB51 or degradation of the antigens, and thus lead to better stimulation of the host immune system.

Although we were able to successfully secrete these antigens using strain RB51, the induction of the immune repose was not as high as when the same antigens were expressed in the cytoplasm i.e not secreted. This was shown by the induction of higher levels of immunoglobulin G and its serotype IgG2a as well as IFN-γ and TNF-α to the cytoplasmic antigens than to the

secreted ones. This was also evident by the protection provided by these constructs, as it was shown that the cytoplasmic form of the antigens provided protection against subsequent challenge with *M. tuberculosis*, whereas the secreted form of the antigens did not provide any level of protection. Contrary to the assumption that secreted antigens of *M. tuberculosis* are central to the induction of protective immunity, this was not the case with strain RB51 expression. Cytoplasmic expression of mycobacterial-secreted antigens was central to the induction of protective immunity against *M. tuberculosis*.

The lack of induction of protective immunity in the secreted form of the antigens is maybe attributed to the antigen-processing pathway. Previous studies with other pathogens have shown that secreted proteins, after being exported to the cytosol of the infected cells, get presented to the immune system mostly via major histocompatibility complex I (MHC-I) pathway [21, 22]. When such proteins are processed through MHC-I molecules, they are presented to cytotoxic T lymphocytes (CTLs) allowing the immune system to scrutinize ongoing intracellular production of these proteins [23]. However, studies of the immune response to TB infections in humans and animal models have shown that $CD4^+$ is undoubtedly the major effector cell in CMI [24, 25], and therefore control of tuberculosis infection requires

$CD4^+$ T cell responses and MHC-II processing of mycobacterial antigens [26]. Therefore, mycobacterial antigens processed via MHC-II would lead to better stimulation of protective immunity than when the same antigens are processed via MHC-I.

None of the individual strains of RB51 carrying mycobacterial antigens was able to elicit significant level of protective immunity on their own. However, when the two strains of RB51 carrying Ag85B and Rv2660c-ESAT6 were combined, a significant protection level was achieved against *M. tuberculosis* challenge. This was consistent with the data that vaccination with a combination of the mycobacterial antigens Ag85B, ESAT6 and Rv2660c leads to better protection than when the same antigens are given individually [8, 9, 27, 28]. This use of a multi-subunit antigen cocktail in a single vaccine formulation is necessary to enhance the chances of covering most MHC types as combining several antigens may have led additive or even synergistic effects [29].

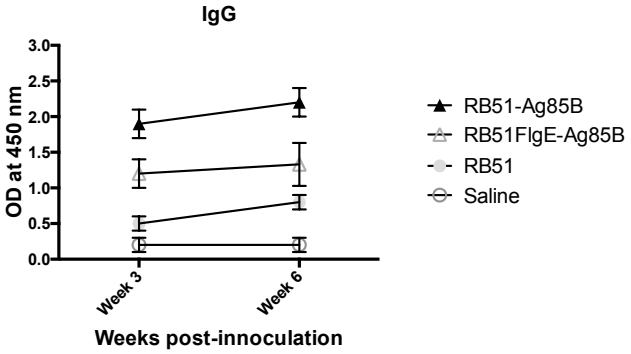
Recent reports from a number of vaccine studies have shown that prime-boost protocols of immunization provide an effective strategy to promote long-term memory and strong cellular Th1 responses against *M. tuberculosis* challenge [30-34]. In an attempt to test this strategy, another TB protection

study was conducted where mice were primed with the two strains of RB51 carrying Ag85B and Rv2660c-ESAT6, and later boosted with a subunit vaccine consisting of Ag85B and ESAT6 emulsified in DDA-MPL adjuvant formulation. The protection level conferred by this vaccination strategy was essentially the same as the protection conferred by BCG, but was higher than the protection level conferred by the subunit vaccine alone. This indicates that RB51 strain carrying mycobacterial antigens could potentially be used to prime the immune system and that a single boost with subunit vaccine is sufficient to confer protective immunity against *M. tuberculosis* challenge similar to that conferred by BCG.

Just as important, expression of mycobacterial antigens in strain RB51 does not compromise its protective efficacy against *B. abortus* 2308 challenge. The *B. abortus* protection studies conducted showed that the recombinant RB51 vaccine strains induced protection against *B. abortus* 2308 challenge at levels similar to those induced by vaccine strain RB51, indicating that the expression of the heterologous antigens did not alter the protective efficacy of the strain. As a matter of fact, higher levels of IFN-γ were secreted by splenocytes against heat-killed RB51 in mice vaccinated with RB51 strains carrying mycobacterial antigens than with mice vaccinated with RB51 alone (Figure 4.4).

In conclusion, we have shown that recombinant RB51 strains expressing mycobacterial protective antigens result in stimulation of antigens specific immune response without altering the vaccine efficacy in protecting against the more virulent strain of *B. abortus* 2308. These recombinant vaccines could potentially be used to protect against *M. tuberculosis* infection.

A



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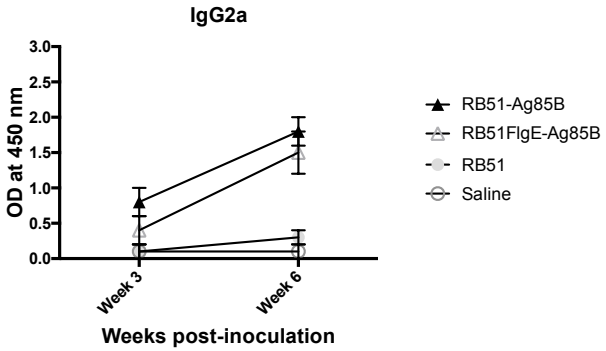

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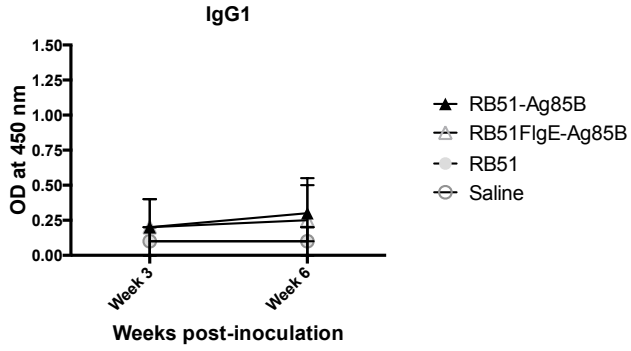
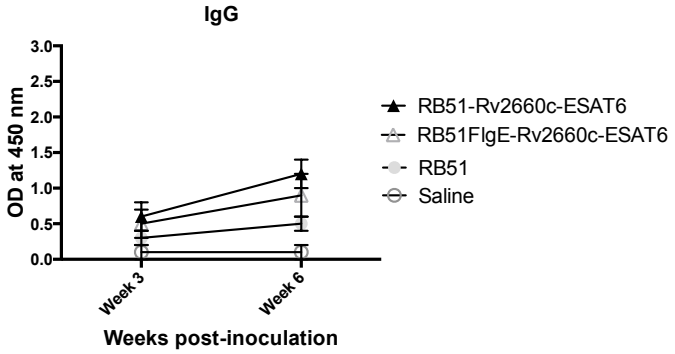


Figure 4.1: ELISA detection of Ag85B-specific IgG (A), IgG2a (B) and IgG1(C) antibodies in serum of mice vaccinated with strain RB51, RB51Ag85B, RB51FlgE-Ag85B or inoculated with saline alone. Sera collected from three mice of each group at 3 and 6 weeks post-vaccination were diluted 1:100 and assayed for the presence of specific antibodies. Results were shown as the means ± S.D. of OD450 of the color developed.

A



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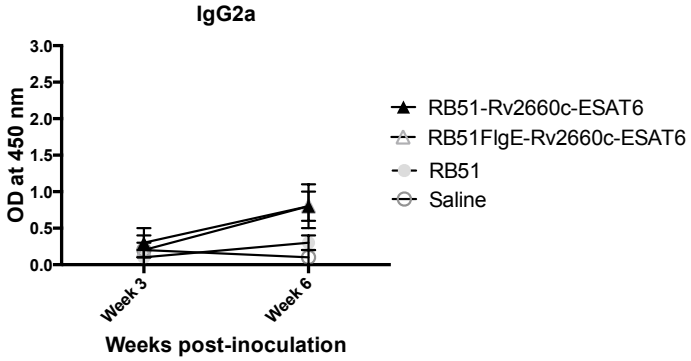

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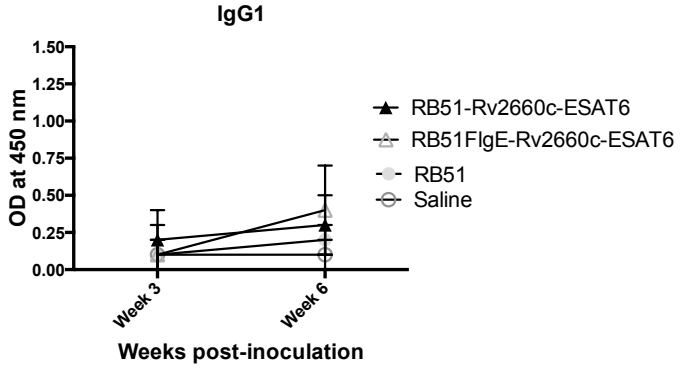
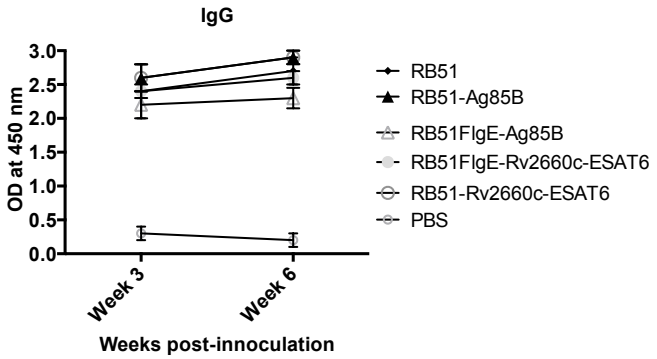


Figure 4.2: ELISA detection of ESAT6-specific IgG (A), IgG2a (B) and IgG1 (C) antibodies in serum of mice vaccinated with strain RB51, RB51-Rv2660c-ESAT6, RB51FlgE-Rv2660c-ESAT6 or inoculated with saline alone. Sera collected from three mice of each group at 3 and 6 weeks post-vaccination were diluted 1:50 and assayed for the presence of specific antibodies. Results were shown as the means ± S.D. of OD_{450} of the color developed.

A



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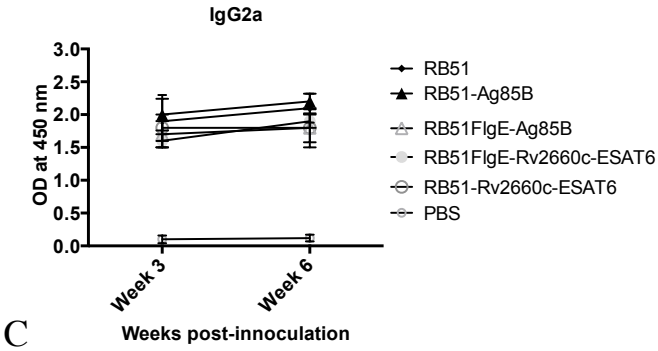

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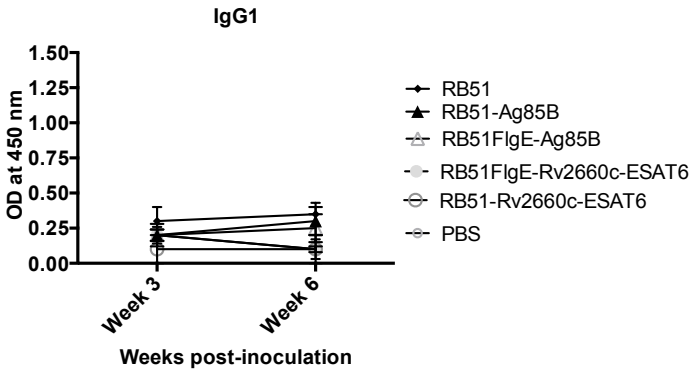

Figure 4.3: ELISA detection of strain RB51 -specific IgG (A), IgG2a (B) and IgG1 (C) antibodies in serum of mice vaccinated with strain RB51, RB51-Ag85B, RB51FlgE-Ag85B, RB51-Rv2660c-ESAT6, RB51FlgE-Rv2660c-ESAT6 or inoculated with saline alone. Sera collected from three mice of each group at 3 and 6 weeks post-vaccination were diluted 1:100 and assayed for the presence of specific antibodies. Results were shown as the means ± S.D. of OD_{450} of the color developed.

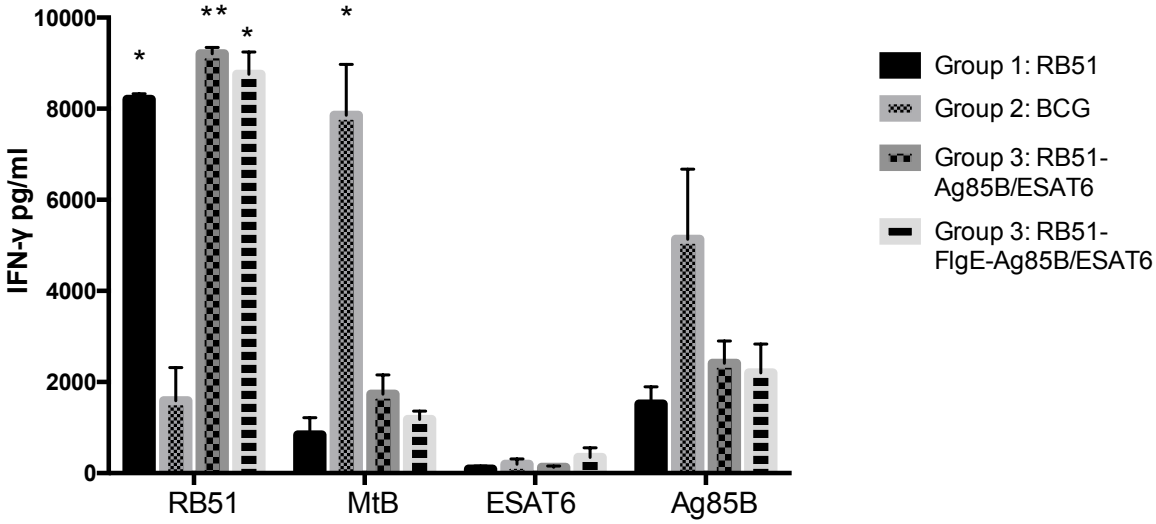

Figure 4.4: Production of IFN-γ by splenocytes from naïve mice and mice vaccinated with strains RB51, BCG, and RB51-Ag85B/Rv2660c-ESAT6, RB51-Ag85B/Rv2660c-ESAT6 after *in vitro* stimulation with heat-killed RB51, heat-killed *M. tuberculosis,* Ag85B and ESAT6.

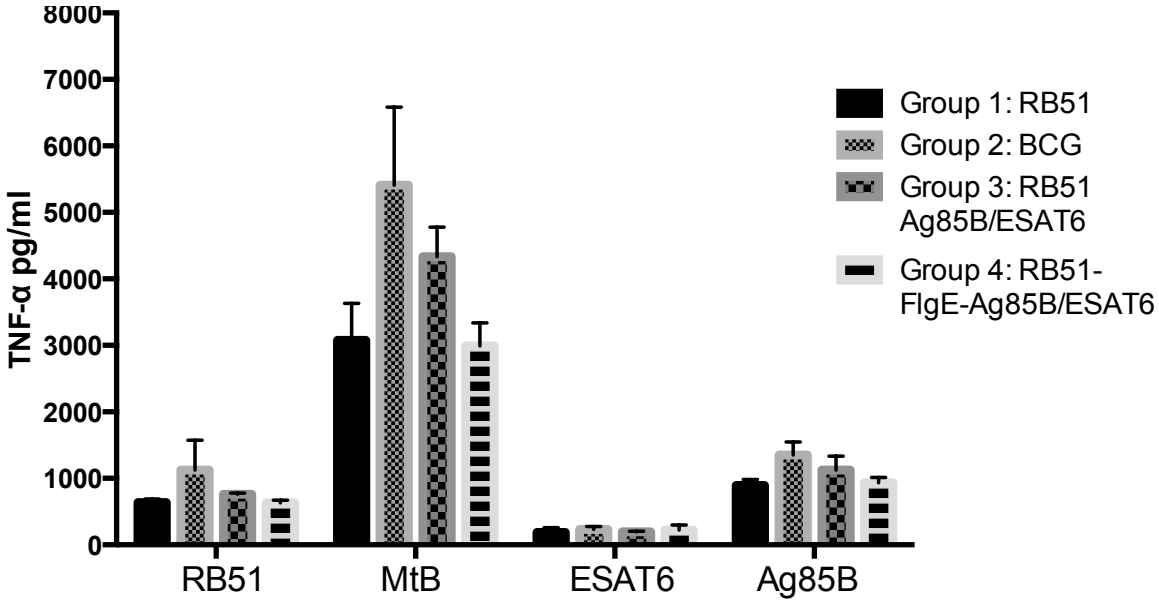

Figure 4.5: Production of TNF-α by splenocytes from nave and mice vaccinated with strains RB51, BCG, and RB51-Ag85B/Rv2660c-ESAT6, RB51-Ag85B/Rv2660c-ESAT6 after *in vitro* stimulation with heat-killed RB51, heat-killed *M. tuberculosis,* Ag85B and ESAT6.

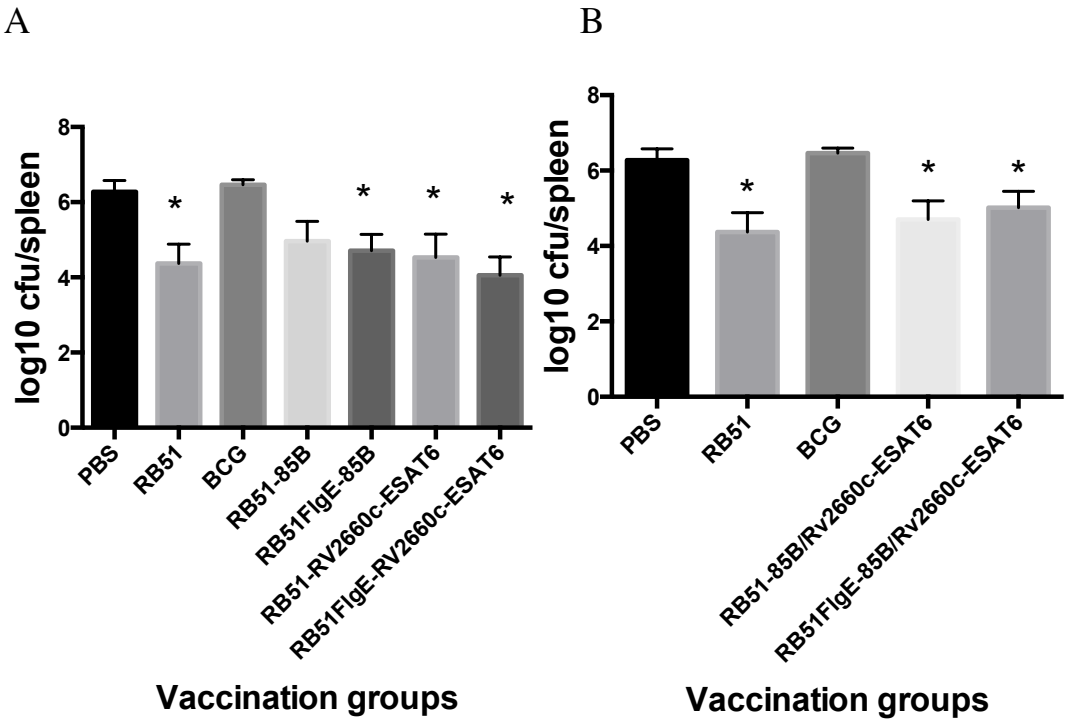

Figure 4.6: Resistance to *B. abortus* strain 2308 challenge infection in mice vaccinated with strains RB51, BCG, and RB51-Ag85B, RB51FlgE-Ag85B, RB51-Rv2660c-ESAT6, and RB5FlgE-Rv2660c-ESAT6 (Figure 4.6A). Resistance to *B. abortus* strain 2308 challenge infection in mice vaccinated with strains RB51, BCG, and combined vaccines RB51-Ag85B/Rv2660c-ESAT6 and RB5FlgE-Ag85B/Rv2660c-ESAT6 (Figure 4.6B). Mice were vaccinated 6 weeks prior to the challenge infection. Two weeks post-challenge infection, the number of strain 2308 CFUs in their spleens was determined. Vaccine constructs with significant level of protection are marked with an asterisk ($P < 0.05$). No significant difference was found between PBS and BCG groups.

A B

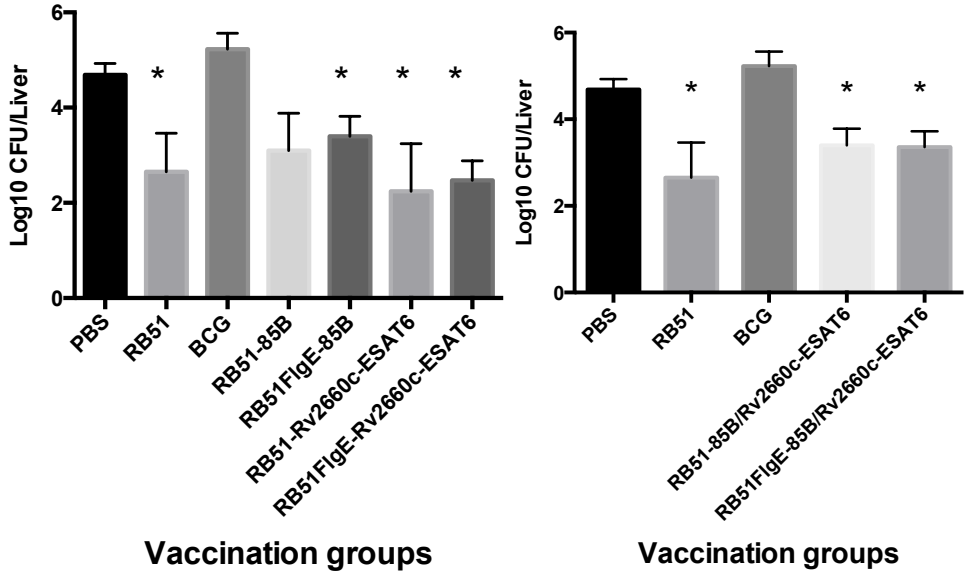

Figure 4.7: Resistance to *B. abortus* strain 2308 challenge infection in mice vaccinated with strains RB51, BCG, and RB51-Ag85B, RB51FlgE-Ag85B, RB51-Rv2660c-ESAT6, and RB5FlgE-Rv2660c-ESAT6 (Figure 4.6A). Resistance to *B. abortus* strain 2308 challenge infection in mice vaccinated with strains RB51, BCG, and combined vaccines RB51-Ag85B/Rv2660c-ESAT6 and RB5FlgE-Ag85B/Rv2660c-ESAT6 (Figure 4.6B). Mice were vaccinated 6 weeks prior to the challenge infection. Two weeks post-challenge infection, the number of strain 2308 CFUs in their lungs was determined. Vaccine constructs with significant level of protection are marked with an asterisk ($P < 0.05$). No significant difference was found between PBS and BCG groups.

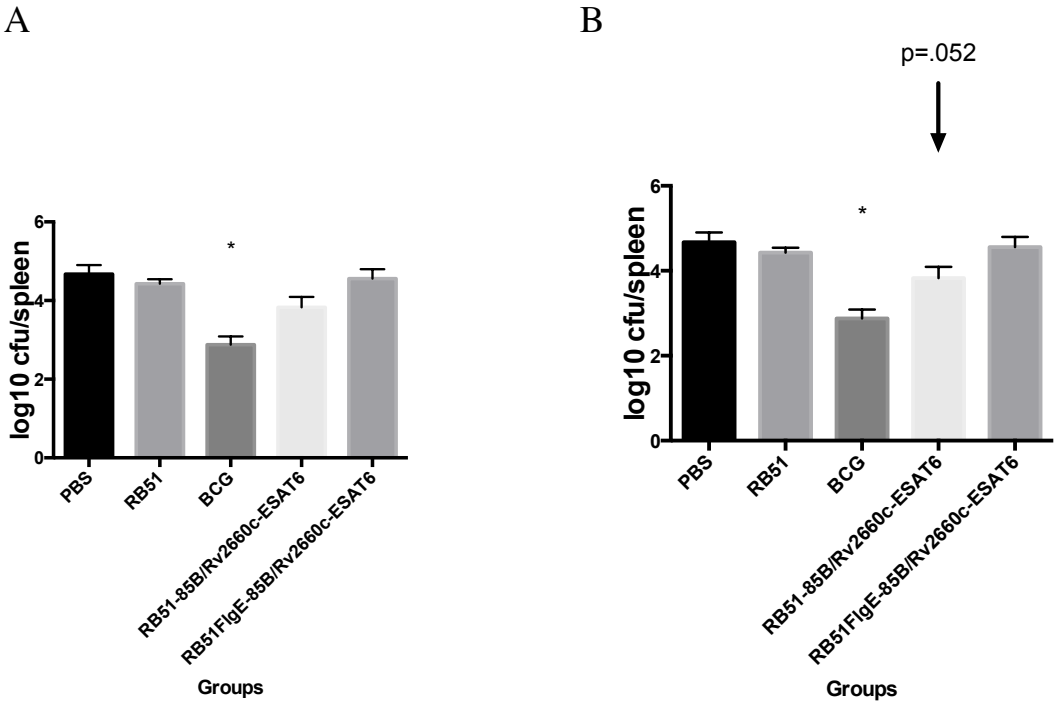

Figure 4.8: Resistance to *M. tuberculosis* challenge infection in mice vaccinated with strains RB51, BCG, and RB51-Ag85B, RB51FlgE-Ag85B, RB51-Rv2660c-ESAT6, and RB5FlgE-Rv2660c-ESAT6 (Figure 4.6A). Resistance to *M. tuberculosis* challenge infection in mice vaccinated with strains RB51, BCG, and combined vaccines RB51-Ag85B/Rv2660c-ESAT6 and RB5FlgE-Ag85B/Rv2660c-ESAT6 (Figure 4.6B). Mice were vaccinated 8 weeks prior to the challenge infection. Four weeks post-challenge infection, the number of *M. tuberculosis* CFU in their spleens was determined. Vaccine constructs with significant level of protection are marked with an asterisk ($P < 0.05$). No significant difference was found between PBS and any of the RB51 groups. RB51-Ag85B combined with RB51-Rv2660-ESAT6 provided the best protection among all RB51 vaccine strains. However, the P value was 0.052.

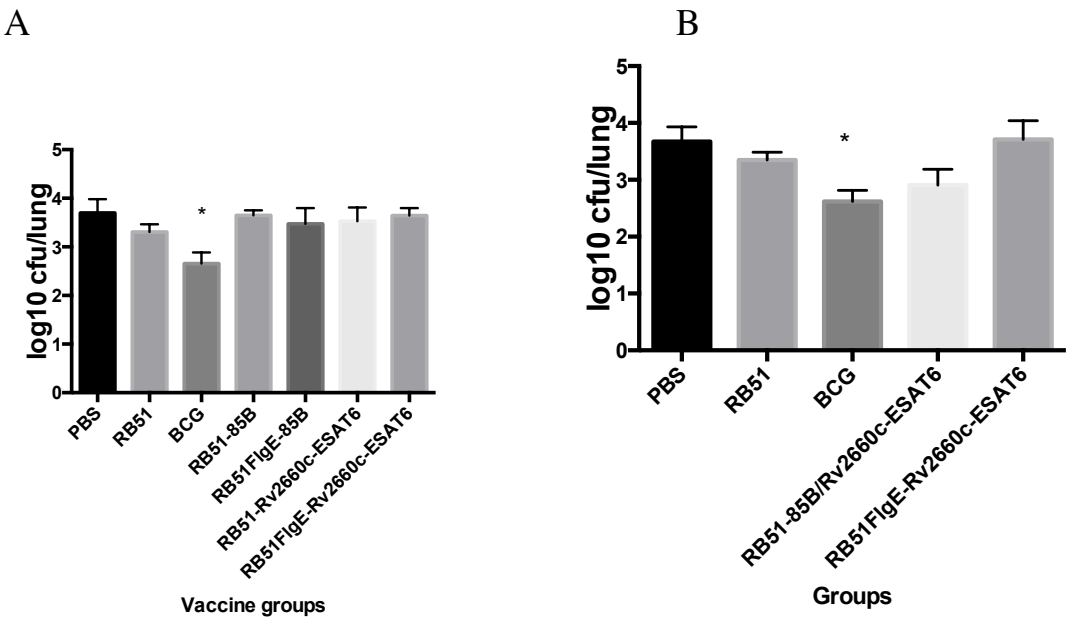

Figure 4.9: Resistance to *M. tuberculosis* challenge infection in mice vaccinated with strains RB51, BCG, and RB51-Ag85B, RB51FlgE-Ag85B, RB51-Rv2660c-ESAT6, and RB5FlgE-Rv2660c-ESAT6 (Figure 4.6A). Resistance to *M. tuberculosis* challenge infection in mice vaccinated with strains RB51, BCG, and combined vaccines RB51-Ag85B/Rv2660c-ESAT6 and RB5FlgE-Ag85B/Rv2660c-ESAT6 (Figure 4.6B). Mice were vaccinated 8 weeks prior to the challenge infection. Four weeks post-challenge infection, the number of *M. tuberculosis* CFU in their lungs was determined. Vaccine constructs with significant level of protection are marked with an asterisk ($P < 0.05$). No significant difference was found between PBS and any of the RB51 groups. RB51-Ag85B combined with RB51-Rv2660-ESAT6 provided the best protection among all RB51 vaccine strains. However, the difference was not significant.

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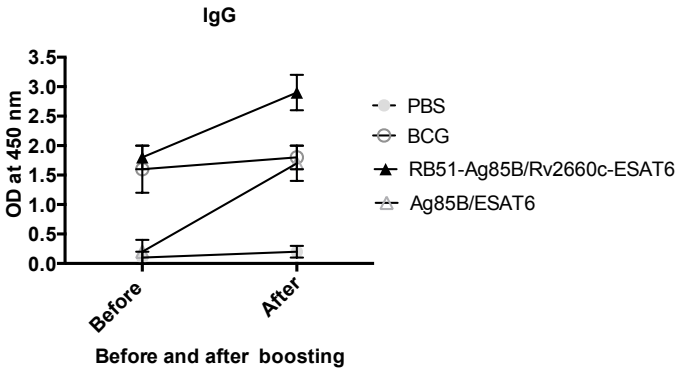

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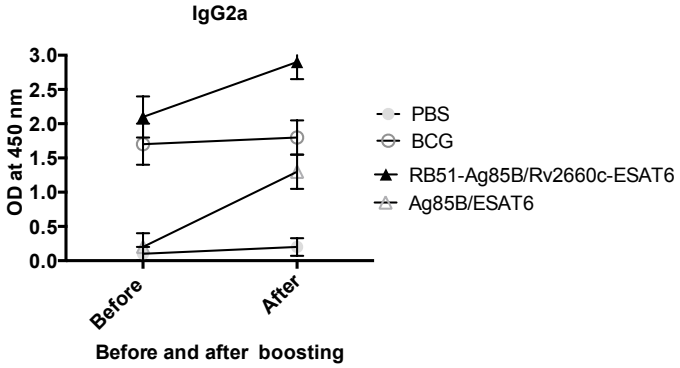

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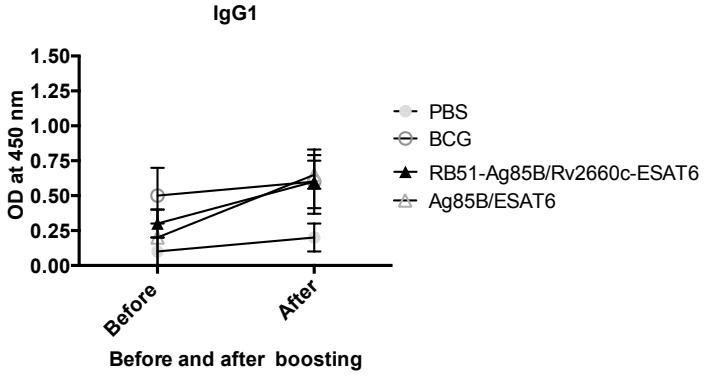

Figure 4.10: ELISA detection of Ag85B-specific IgG (A), IgG2a (B) and IgG1(C) antibodies in serum of mice vaccinated BCG with combined strains of RB51 carrying Ag85B and Rv2660c-ESAT6 or inoculated with saline alone. Sera collected from each group at before and after boosting with Ag85B and ESAT6. Sera were diluted 1:100 and assayed for the presence of specific antibodies. Results were shown as the means ± S.D. of OD_{450} of the color developed.

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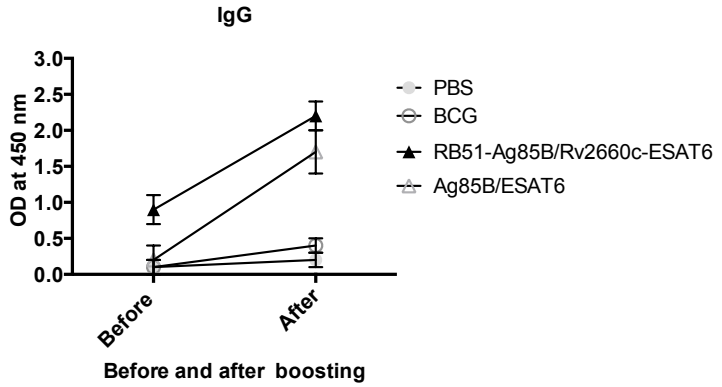

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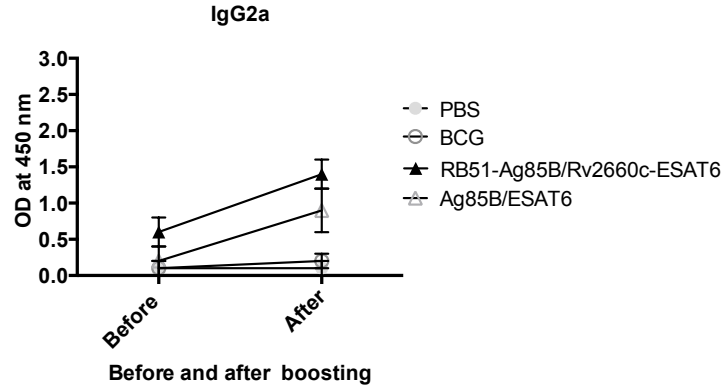

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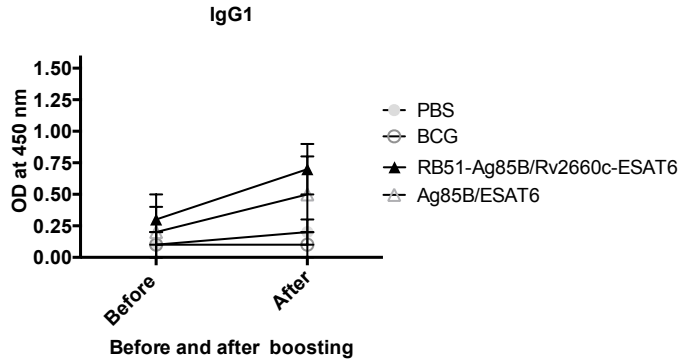

Figure 4.11: ELISA detection of ESAT6-specific IgG (A), IgG2a (B) and IgG1(C) antibodies in serum of mice vaccinated BCG with combined strains of RB51 carrying Ag85B and Rv2660c-ESAT6 or inoculated with saline alone. Sera collected from each group at before and after boosting with Ag85B and ESAT6. Sera were diluted 1:50 and assayed for the presence of specific antibodies. Results were shown as the means ± S.D of OD450 of the color developed.

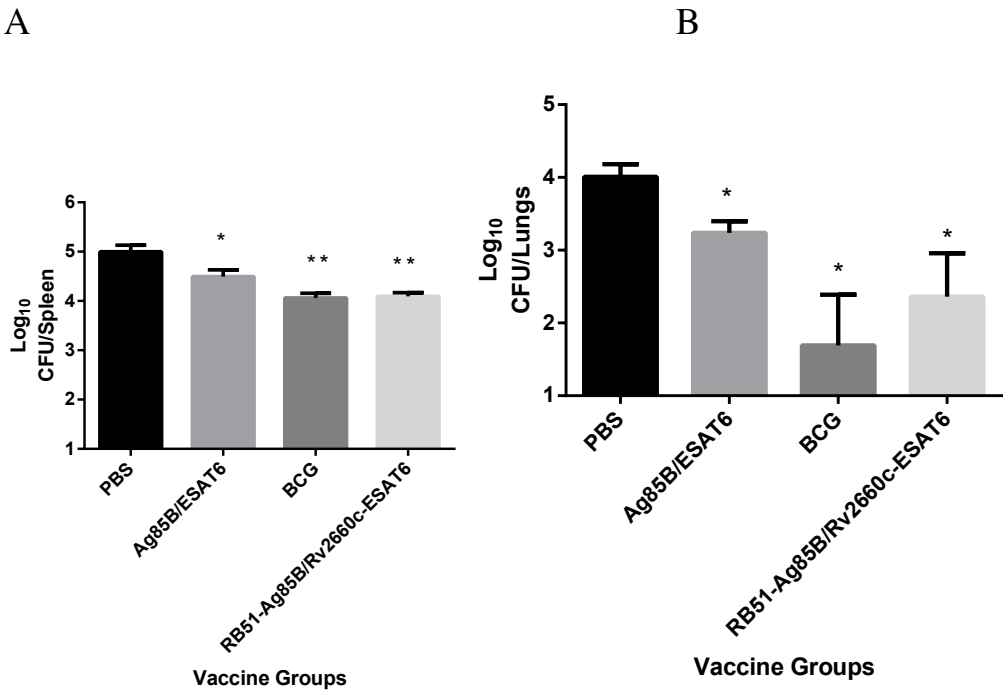

Figure 4.12: Resistance to *M. tuberculosis* challenge infection in mice vaccinated with BCG, and combined strains of RB51 carrying Ag85B and Rv2660c-ESAT6, and Ag85B/ ESAT6 subunit vaccine. Mice were vaccinated for 8 weeks then boosted with subunit vaccines 2 weeks prior to the challenge infection. Four weeks post-challenge infection, the number of *M. tuberculosis* CFUs in the spleens (Figure 4.12A) and lungs (Figure 4.12B) were determined. Vaccine constructs with significant level of protection are marked with one asterisk ($P < 0.05$). Vaccine constructs marked with two asterisks provide significantly better protection than mice vaccinated with subunit vaccines only.

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Chapter 5

Discussion, Conclusion and Future Work

Discussion and Conclusion

Tuberculosis is one of the most common infectious diseases and the greatest cause of death by an infectious agent worldwide [112]. The lethality of TB is partly due two main reasons: First, its dynamic interaction with the HIV pandemic with one out of four TB deaths found to be HIV-related. Second, the increasing incidence of multidrug-resistant strains of *M. tuberculosis*. This increasing prevalence of multidrug-resistant strains of *M. tuberculosis*, coupled with the highly infectious nature of the disease and the lack of an effective vaccine make TB a significant public health issue worldwide. Therefore, there is a dire need for an improved second-generation vaccine that can prevent the initial establishment of TB, and prevent dissemination of the disease to unaffected individuals.

Although recent advancements in bacterial vaccine technology have lead to effective vaccines against many pathogens, the immunity conferred by these vaccines have been dependent on humoral immunity, which is sufficient to protect against many extracellular pathogens [113]. However, it has proven challenging to develop prophylactic vaccines against intracellular pathogens,

such as *M. tuberculosis,* where control depends primarily on cell-mediated immunity [11].

The attenuated *M. bovis* BCG vaccine is currently the only TB vaccine approved for human use, but its efficacy remains controversial [52]. In an attempt to generate a vaccine against tuberculosis, we hypothesized that *B. abortus* vaccine strain RB51 expressing mycobacterial antigens could result in a prophylactic vaccine against tuberculosis. Strain RB51 was chosen based on it safety and protective efficacy which have been demonstrated under laboratory and field conditions in animals [114, 115]. Additionally, protection afforded by strain RB51 vaccination is due to induction of cell-mediated immunity, more specifically through production of IFN-γ [105, 116, 117]. More importantly, studies have shown that immunization of mice with recombinant strains of RB51 expressing heterologous proteins induces a Th1 type of immune responses specific to the expressed protein without altering efficacy against experimental challenge with *B. abortus* [105, 107, 108]. These findings provide a rationale for the use of strain RB51 as a prime vaccine candidate for the delivery of protective antigens of *M. tuberculosis*, in which cell-mediated immunity is essential for protection. In order to test this hypothesis, a series of experiments were designed to construct multiple recombinant strains of RB51 that could express as well as

secrete mycobacterial protective antigens Ag85B, ESAT6 and Rv2660c. The protective efficacy of the recombinant strains was then tested in a murine model against brucellosis and tuberculosis infections.

The research presented in this dissertation has been divided into three main chapters. The main focus of chapter two was to strengthen heterologous protein expression and to modify the intracellular localization or the mode of antigen delivery in strain RB51. Expression vector pNS4 was the vector of choice for cloning and expressing heterologous proteins in strain RB51. This expression vector does not code for an antibiotic selective marker for plasmid stability, instead it carries the *leuB* gene that complements a leucine auxotroph strain of RB51 (RB51*leuB*-), which was designed in our laboratory previously [118, 119]. The use of this strain of RB51 along with pNS4 averts the introduction of antibiotic resistance genes into a live attenuated vaccine, and therefore provides a safe vaccine platform.

In order to strengthen the expression of proteins in strain RB51, the *groE* promoter in pNS4 expression vector was replaced with *trcD* promoter, one of the strongest semisynthetic promoters ever developed for *Brucella* species [120]. Additionally, signal sequences of three *Brucella* proteins, which were shown to be either cell-membrane bound or secreted, were designed

downstream the promoter to direct the expressed heterologous protein into the culture supernatant. The rationale for the use of signal sequences was the thought that secretion of *M. tuberculosis* protective antigens would result in a better protection. This hypothesis was based on the fact that the *M. tuberculosis* antigens expressed in strain RB51, Ag85B and ESAT6, are naturally secreted by *M. tuberculosis* [84, 121]; and therefore secretion of such antigens using strain RB51 would provide a similar mode of antigen delivery. Green fluorescent protein (GFP) was used as a reporter protein to test the efficacy and stability of the newly designed expression vectors. Using GFP, we were able to show that newly designed vectors were able to express and secrete heterologous proteins in strain RB51 without any stability issues.

The focus of chapter three was to test the efficacy of the newly designed expression vectors to express and secrete mycobacterial protective antigens Ag85B, ESAT6 and Rv2660c from *M. tuberculosis*. These protective antigens were chosen based on their ability to elicit protective immunity against TB challenge as documented previously [56, 57, 74, 75, 80, 84, 90-92, 121, 122]. The genes coding for these three mycobacterial antigens were cloned into pNS4TrcD (non-secreting) and pNS4TrcD-FlgE (secreting) expression vectors. Upon transformation into strain RB51, much to our

surprise, no expression of recombinant proteins was achieved. As recombination events took place within the coding sequences of the protective antigens to form unrecognized chimera. The mutations were attributed to the difference in codon usage between *Mycobacterium* species and *Brucella* species. Expression of the recombinant proteins was only achieved upon optimizing their codons for *Brucella* expression. This optimization of codon usage resulted in stable expression vectors and successful expression of *M. tuberculosis* protective antigens in strain RB51 without showing any signs of lethality or other forms of protein toxicity.

The stability of the recombinant strains was then tested *in vitro* using GFP as a reporter protein. Stability results indicated the expression vectors were highly stable when grown on leucine deficient medium. However, when cultured on non-selective medium (TSA), the expression vectors were stable for approximately 4-5 weeks. Since stain RB51 is usually cleared within 5-6 weeks in BALB/c mice, these results were very acceptable and lead us to test the stability of the recombinant vaccines in our murine model. BALB/c mouse was the model of choice. This model is one of the best rodent models that can be used to evaluate both brucellosis and tuberculosis infection [123, 124]. Stability data generated from this animal study indicated that the

recombinant RB51 strains were stable 3-6 weeks post vaccination with no signs of recombination events or loss of protein expression as indicated by DNA sequencing and western blotting of isolates recovered from sacrificed animals. More importantly, the expression of mycobacterial protective antigens did not alter the attenuation level of the recombinant strains, since all vaccine strains were cleared from mice by approximately 6 weeks post vaccination, the same amount of time required for clearance of vaccine strain RB51.

The focus of chapter 4 was to test the efficacy of the recombinant strains of RB51 in inducing antigen-specific immune response that could lead to protective immunity against a challenge by wild-type *M. tuberculosis* and *B. abortus* strains. Two sets of immunology experiments representing antibody-mediated and cell-mediated immunity were carried out. In the first set, the quality and strength of the immune response induced by the recombinant vaccines were measured. This was done by measuring the level of IgG and its isotypes IgG1 and IgG2s against mycobacterial protective antigens Ag85B, ESAT6 and heat-killed RB51 in mice vaccinated with each recombinant strain of RB51. Serological analysis indicated that mice vaccinated with each recombinant strain developed antigen-specific IgG antibodies to Ag85B, ESAT6 and heat-killed RB51 of predominantly an

IgG2a isotype. However, higher levels of antibodies were detected in mice vaccinated with RB51 strains expressing the non-secreted form of the antigens compared to mice vaccinated with RB51 strains expressing the secreted form of the antigens.

The second set of studies involved antigen-specific cytokine expression assay. Analysis of the cytokine profile of vaccinated mice showed production of antigen-specific IFN-γ and TNF-α in splenocyte culture supernatant from mice vaccinated with the recombinant strains upon stimulation with mycobacterial antigens. The generation of an antigen-specific immune response characterized by the induction of IFN-γ, TNF-α and IgG2a polarized antibodies in the absence of IL-4 and little-to-no IgG1 antibodies indicate a polarization towards a Th1 type of response, a pivotal step for the control of TB infection [11, 55, 64, 78, 79, 125, 126].

The protective efficacy of the recombinant vaccines was then measured in a BALB/c mouse model against *B.abortus* 2308 challenge. In general, the recombinant RB51 strains expressing mycobacterial antigens remained protective against *Brucella* infection indicating that expression of heterologous antigens in strain RB51 did not significantly impair the ability of the vaccine to mount protective immunity to *Brucella* infection. This was

a vital step in the generation of an effective and useful RB51-based bivalent vaccine.

In the *M. tuberculosis* protection study, none of the individual strains were able to confer protection against *M. tuberculosis* challenge, but the combination of two RB51 vaccine strains expressing Ag85B and fusion Rv2660c-ESAT6 antigens lead to significant level of protection. However, the protection level was less than that conferred by BCG. The combination of two strains RB51 expressing the non-secreted mycobacterial antigens resulted in better level of protection that the secreted form. This was consistent with the immune response generated earlier in the immunology studies, i.e higher levels of IFN-γ, TNF-α and IgG2a antibodies were detected in mice vaccinated with RB51 strains expressing the non-secreted antigens than with the secreted ones.

In order to enhance the protection level conferred by the combination vaccines, RB51-Ag85B and RB51-Rv2660c-ESAT6, a second *M. tuberculosis* protection study was carried out. In this study, mice were primed with the RB51 recombinant strains and later boosted once with Ag85B and ESAT6 emulsified in DDA-MPL adjuvant formulation. This combination of adjuvants has been shown to invoke a sufficient cell-

mediated immune response against subunit vaccines [127-129]. Serological analysis from these mice showed increased levels of IgG2a production in mice vaccinated with RB51 strains expressing Ag85B and ESAT6 after boosting with subunit vaccine. This increase in production of IgG2a, which is indicative of a Th1 profile, resulted in protection levels similar to that conferred by BCG upon challenging with *M. tuberculosis*. These results clearly suggest that RB51-based recombinant vaccine could confer protection against tuberculosis infection in our animal model.

Future Directions

Our data warrant further refinement of strain RB51 as a platform for the development of a bivalent vaccine against brucellosis and tuberculosis in humans. For use in humans, a vaccine must be safe (i.e. not produce disease or more than minimal local or systemic reactions) and provide a long-lived protection [130]. Although we have shown recombinant RB51 strains to be efficacious against brucellosis and tuberculosis infection, potential safety issues with the use of live attenuated *Brucella* vaccines in humans includes failure to clear the vaccine strain and/or the possibility that the vaccine strain might cause disease[130, 131].

One approach to address safety issues involving live vaccines is the use of

gamma-irradiation to further attenuate and create metabolically-active but non-viable vaccine for human use. Studies have demonstrated that exposure of *Brucella* to certain levels of gamma-irradiation leads to inability of the bacteria to replicate *in vitro* without terminating its metabolic activity [132]. By maintaining metabolic activity, the irradiated bacteria can mimic natural pathogenesis of live bacteria [133, 134].

This technique has been applied to strain RB51. In a study by Sanakkayala and colleagues [110], it was shown that gamma-irradiation of strain RB51 resulted in complete loss of replicative ability, however the strain remained metabolically active. Additionally, the study showed that irradiation of strain RB51 did not alter its antigenicity or its ability to induce secretion of IFN-γ, and as a result, the protection level conferred by the irradiated strain was similar to that conferred by the live vaccine. Just as important, gamma-irradiation of a recombinant strain of RB51 expressing LacZ did not effect the induction of LacZ-specific immune response, thus, indicating that the generation of antigen-specific immune response to heterologous protein expressed in strain RB51 is not altered by gamma-irradiation [110].

Future studies should also include creation of rifampicin susceptible RB51 strains to serve as a platform for a bivalent vaccine against both brucellosis

and tuberculosis. Such refinements would negate some concerns associated with the use of strain RB51 in humans without affecting the strain's ability to induce immune responses and protective immunity against *Brucella* antigens or heterologous proteins.

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