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## Design and Humoral Analysis of Two Epitope-Based *Brucella abortus* DNA Vaccines

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DESIGN AND HUMORAL ANALYSIS OF TWO EPITOPE-BASED BRUCELLA ABORTUS  
DNA VACCINES

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
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## LIST OF ABBREVIATIONS

APC –	Antigen presenting cell
bp –	Base pairs
CD –	Cluster of differentiation
CFU –	Colony forming units
CHO –	Chinese hamster ovary
CpG –	CpG oligodeoxynucleotide
i.p. –	Intraperitoneal
i.g. –	Intragastric
IFN- $\gamma$ –	Interferon- $\gamma$
IL –	Interleukin
IV	Intravenous
LPS –	Lipopolysaccharide
mAb –	Monoclonal antibody
MHC I–	Major histocompatibility complex class I
MHC II –	Major histocompatibility complex class II
NO –	Nitric oxide
Omp –	Outer membrane protein
OPS –	O-polysaccharide
PBMC –	Peripheral blood monocytes
RB51 –	<i>Brucella abortus</i> Strain RB51
RB51SOD –	<i>Brucella abortus</i> Strain RB51 SOD overexpression strain
ROS	Reactive oxygen species
Th –	T helper cell
TLR –	Toll like receptor
TNF- $\alpha$ –	Tumor necrosis factor- $\alpha$
TNFR –	Tumor necrosis factor receptor
TGF- $\beta$ –	Transforming growth factor- $\beta$
SOD –	Superoxide dismutase
WT –	Wild type



## ABSTRACT

*Brucella* is a genus of Gram negative, facultative intracellular pathogens which cause brucellosis, one of the most wide spread zoonotic diseases. Brucellosis causes a severe economic burden due to reproductive loss in animals and human infection. Vaccination of animals has proven to be the most effective means of controlling brucellosis; however the current live-attenuated vaccines are not considered ideal. The live-attenuated vaccines *Brucella abortus* Strain 19 and RB51 remain pathogenic to humans, and the former interferes with diagnostic tests due to induction of antibodies against the O-polysaccharide. DNA vaccination with single antigens has proven to be successful at protecting mice against *B. abortus* challenge, however this method is less effective in large animals. Immunization with a combination of antigens has been shown to provide more protection than single antigens.

In an attempt to develop a better DNA vaccine, two multivalent multi-epitope plasmids were constructed using known protective antigens and bioinformatics technologies. Epitopes predicted to induce cell-mediated immunity were selected from Cu/Zn superoxide dismutase, outer membrane protein (Omp) 16, Omp19, ribosomal subunit L7/L12, BP26, ribosomal subunit L9, and Omp25. The plasmids were transfected into Chinese hamster ovary (CHO K1) cells, and PCR was used to confirm presence of the sequences in the genome. The transcription of the *BabV1* and *BabV2* genes to RNA was confirmed using RT-PCR. Finally, Western blots using sera from Strain 19 infected goats suggest the protein is not recognized by the humoral response of vaccinated animals. Further research is required to determine if the p425/BabV1 and p425/BabV2 vaccines are recognized by the cell-mediated immune response of infected or vaccinated animals.

## CHAPTER I LITERATURE REVIEW

### INTRODUCTION

*Brucella* is a genus of Gram negative, facultative intracellular pathogens which cause brucellosis, one of the most widespread global zoonotic diseases [1, 2]. Infection in cattle causes abortion and infertility; and in humans, undulant fever, arthritis, endocarditis and spondylitis [3, 4]. The *Brucella* spp. are named based on their natural host with *B. melitensis* for goats, *B. abortus* for cattle, *B. suis* for swine, *B. canis* for dogs, *B. ovis* for sheep, and *B. neotomae* for desert mice [5]. Smooth *Brucella* species express full lipopolysaccharide (LPS) molecules, while rough *Brucella* spp. lack O-polysaccharide antigen (OPS) of the LPS [6, 7]. The brucella OPS is a homopolymer of 100 residues of 4-formamido-4, 6-dideoxymannose linked by predominately  $\alpha$ -1,2 bonds in A epitope strains or  $\alpha$ -1,3 bonds every fifth residue in M epitopes strains [8]. *Brucella* spp. are aerobic, fastidious, slow growing, and require supplemental CO<sub>2</sub>, thiamine, nicotinamide, biotin, and serum for growth [9, 10].

Humans may be exposed by ingestion of unpasteurized milk from infected animals; contact with infected tissue, blood, lymph; or inhalation of aerosols [2, 6, 11]. Globally, there are approximately 500,000 cases of human brucellosis reported each year resulting in high economic burdens due to animal brucellosis in developing countries [12, 13]. One of the most effective means of controlling brucellosis, in both humans and animals, is vaccination of animals [14, 15]. The live-attenuated vaccines *B. abortus* Strain 19 and rough *B. abortus* Strain 51 (RB51) grant protection against abortion and infection caused by *B. abortus* in cattle, and the live-attenuated vaccine Rev. 1 protects against *B. melitensis* in goats and sheep [5, 14]. Though they are effective in protecting against brucellosis, these are not considered ideal vaccines due to

residual virulence in humans; ability to cause abortion in pregnant animals; and in smooth strains, such as Strain 19 and Rev. 1, induction of anti-OPS antibodies that interfere with serological tests [14, 16]. There is currently no vaccine available for use in humans or wildlife reservoirs [17].

The goal of this research project was to determine if it is possible to design a DNA vaccine recognized by the humoral immune response of vaccinated animals. A combination of literature mining and bioinformatics technologies was used to select protective epitopes against *B. abortus* infection. Plasmids coding for these epitopes were constructed and transfected into Chinese hamster ovary (CHO K1) cells. The CHO K1 genome was then analyzed via PCR to determine the presence of the vaccine sequences. Transcription of the vaccine sequence into RNA was determined via RT-PCR. Finally, media and cell lysate from the CHO K1 cells were analyzed via Western blot using Strain 19 vaccinated goat sera to detect the recombinant proteins.

## **ADAPTIVE IMMUNE RESPONSE**

The immune system is divided into the innate and adaptive systems [18]. The innate immune system is the first line of defense against infection and has no immunological memory [18]. On primary exposure, the adaptive immune system develops a specific memory response that is recalled on subsequent exposures [18]. The adaptive immune system is divided into cell-mediated, which consists of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and humoral immunity, which consists of B cells and antibodies. Vaccines induce an adaptive immune response which is recalled on secondary exposure. During the development of an adaptive cell-mediated response, naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells differentiate and proliferate when stimulated by cytokines, costimulatory molecules, and antigen presenting cells (APC) displaying an epitope in the context

of Major Histocompatibility Complex (MHC) molecules recognized by the T cell receptor [19, 20]. The cytokines and costimulatory molecules that the naïve T cells are exposed to during differentiation determine the effector function of the differentiated cell [19, 21]. A number of these differentiated T cells become memory cells which are recalled rapidly on secondary exposure [22].

Interleukin-12 (IL-12) causes naïve CD4<sup>+</sup> cells to differentiate into Type 1 T helper (Th1) cells which secrete IL-18, IL-2, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interferon- $\gamma$  (IFN- $\gamma$ ) [19, 23]. The latter three cytokines are capable of activating macrophages and CD8<sup>+</sup> cells to kill intracellular pathogens, such as *Brucella* spp. [19]. Type 2 T helper (Th2) cells are differentiated by IL-4 stimulation and secrete IL-4, IL-5 and IL-10 [19]. Th2 cells promote antibody, primarily IgG1 and IgE, production by B cells and are therefore considered to be a regulator of the humoral immune response [19]. In addition, IL-2 promotes T cell proliferation and survival; and is important for both Th1 and Th2 differentiation [23-25]. Th17 cells are produced when naïve CD4<sup>+</sup> T cells are stimulated by both IL-6 and transforming growth factor- $\beta$  (TGF- $\beta$ ) and secrete IL-17 [19]. IL-17 is able to activate various cell types to produce pro-inflammatory cytokines and molecules such as IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and nitric oxide (NO) [26].

CD8<sup>+</sup> cells kill infected host cells through secretion of granzyme and perforin as well as secretion of cytokines such as TNF- $\alpha$  and IFN- $\gamma$  [21, 27]. Although the exact mechanisms are unclear, establishment of memory CD8<sup>+</sup> cells and their effector function upon secondary exposure depends on CD4<sup>+</sup> T cell help, antigen avidity, and cytokine stimulation [21, 24, 25]. Indeed, CD8<sup>+</sup> cells that differentiate in the absence of CD4<sup>+</sup> cells during primary infection show reduced proliferation and cytokine secretion [21, 25].

Major Histocompatibility Complex are a class of molecules which bind to and present processed peptide epitopes on the surface of cells for T cell recognition [28, 29]. Epitopes, also known as antigenic determinants, are the minimum essential portions of antigens recognized by the immune system [27, 30]. Linear epitopes are epitopes with amino acid residues adjacent in sequence [31], while conformational epitopes are formed by residues that are discontinuous but close in three dimensional space [32]. MHC I molecules are displayed on the surface of all nucleated cells and present endogenous linear epitopes that are 8-11 amino acids (mostly nonamers) long to CD8<sup>+</sup> T cells [27, 29, 33]. MHC II molecules are displayed on the surface of APCs and present exogenous 13-30 amino acid long epitopes to both CD4<sup>+</sup> T cells (linear epitopes) and B cells (linear and conformational epitopes) [27, 33, 34].

## **TH1 RESPONSE**

Resistance to brucellosis is primarily mediated by a Th1 immune response, mainly due to IFN- $\gamma$  activation of macrophages [5, 17, 35-38]. CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets have also been shown to be involved in resistance against infection by production of cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , and cytotoxic killing of infected macrophages [5, 17, 27, 35, 37-43]. Studies on the depletion and passive transfer of T cell subsets have revealed that both CD4<sup>+</sup> and CD8<sup>+</sup> cells contribute to resistance, and protection is greater when both subsets are present [39, 40].

Exogenous IL-2 in culture with *B. abortus* Strain 19, Strain RB51, and virulent Strain 2308 infected macrophages reduced the replication of bacteria at 24 hours but not at 48 hours [42]. However, addition of IL-2 to IFN- $\gamma$  activated macrophages neither enhanced nor inhibited control of infection [42].

Both CD4<sup>+</sup> and CD8<sup>+</sup> produce IFN- $\gamma$  in response to brucella infection [37]. IFN- $\gamma$  is crucial for the control and clearance of brucellosis in the animal model [37, 38, 44]. IFN- $\gamma$

enhances resistance by increasing the production of reactive oxygen species (ROS) by macrophages, and the amount of IFN- $\gamma$  produced by both murine CD4<sup>+</sup> cells and splenocytes correlates with resistance to brucellosis [38]. Exogenous IFN- $\gamma$  in macrophage culture increased anti-brucella activity, including increased ROS production, and reduced replication in the infected IFN- $\gamma$  activated macrophages [38, 42, 44]. IFN- $\gamma$  neutralization by anti-IFN- $\gamma$  monoclonal antibodies (mAb) and IFN- $\gamma$  gene knock out mice show increased splenic colony forming units (CFU) in both naturally susceptible BALB/c and naturally resistant C57BL/6 mice one week post infection [38, 43, 44]. Both resistant and susceptible IFN- $\gamma$  knock out mice die by six and 12 weeks post infection, respectively [38]. Resistant mice produce IFN- $\gamma$  at all measured time points during infection, while susceptible mice stop producing IFN- $\gamma$  between weeks three and six [38]. During this IFN- $\gamma$  hiatus CD8<sup>+</sup> cells and TNF- $\alpha$  control the infection, but are unable to clear brucellae from the animal [38].

Tumor necrosis factor- $\alpha$  enhances resistance to brucella infection during the first two to three weeks of infection in both wild type (WT) and IFN- $\gamma$  knock out mice [43, 45] however, splenic CFU in TNF- $\alpha$  deficient mice were similar to control mice by six weeks post infection [45]. In both the presence and absence of IFN- $\gamma$ , TNF- $\alpha$  has been shown to enhance macrophage killing of *Brucella* spp., but it is not able to clear brucella infection *in vivo* [38, 43, 46]. Further, mice deficient in TNF receptors (TNFR<sup>-/-</sup>) were less able to control brucellosis and produced negligible quantities of IL-12 from splenic cells of Strain 19 infected C57BL/10 mice in the first two weeks of infection [47], suggesting that TNF- $\alpha$  indirectly promotes the development of a Th1 response [45]. These data suggests that TNF- $\alpha$  plays both a direct and indirect role in resistance to brucellosis in the animal model.

## TH2 RESPONSE

The contribution of IL-4 to brucellosis susceptibility is controversial [48, 49]. The addition of exogenous IL-4 to IFN- $\gamma$  activated macrophages did not inhibit or enhance control of brucella infection *in vitro* [42]. IL-4 deficient C57BL/6 mice, but not BALB/c mice, are more resistant than WT to *B. melitensis* when analyzed on days 5, 12 and 28 post infection [48]. However, IL-4 deficient BALB/c do not show enhanced resistance at these time points [48]. IL-4 neutralization with mAb reduces the splenic CFU of less virulent *B. abortus* Strain 2308 from both BALB/c and C57BL/10 mice but only at lower challenge doses [49]. IL-4 is also an important survival factor for B cells thus generating an antibody response [50]. IL-4 neutralization reduced antibody production and indirectly promoted a Th1 response [49]. This suggests that the deleterious effects of IL-4 are mediated by inhibition of Th1 development and not directly on macrophage killing of *Brucella* organisms.

Interleukin-10 has been shown to increase susceptibility to brucellosis in the mouse model [17, 38, 44, 49]. BALB/c mouse splenocytes and B cells produce significant amounts of IL-10 after infection with *B. abortus* [37, 38]. Addition of IL-10 to brucella-infected macrophage cultures reduced control of infection both in the presence and absence of exogenous IFN- $\gamma$  [44]. Interleukin-10 is also known to suppress IL-12 production, indirectly suppressing the Th1 response and IFN- $\gamma$  production [44, 51, 52]. Taken together, this indicates IL-10 both directly and indirectly increases susceptibility to brucellosis.

## TH17 RESPONSE

The Th17 response is an important mediator of mucosal immunity against both extracellular and intracellular pathogens [53]. However, its role in mucosal and systemic immunity to brucellosis has not yet been clearly defined despite exposure commonly occurring

through mucosal routes [48, 54, 55]. The Th17 response does not appear to have an important role in the primary infection of BALB/c or C57BL/6 mice against *Brucella* spp.; however it has been shown to be protective in the adaptive response of BALB/c mice vaccinated with RB51 [48, 54, 56]. This is consistent with a study that found vaccinating cattle with Strain 19 or RB51 induces Th17 cells and IL-17 production which peaked one year after vaccination [57]. The amount of protection an adaptive Th17 response against brucellosis grants varies based on vaccine, as neutralization of IL-17 with mAb ranges from complete abrogation to only a slight reduction in protection [48, 54, 56]. While evidence suggests that a Th17 response is not necessary for clearance or protection against brucella infection, it is capable of working synergistically with the Th1 response to enhance protection; and IL-17 compensates to a degree for a lack of IFN- $\gamma$  [48, 56].

## **B CELLS**

The effector functions of B cells include antibody production and cytokine secretion [58]. Both BALB/c and C57BL/6 B cell deficient mice clear *Brucella* bacteria more rapidly than WT mice through an antibody independent mechanism [37]. Indeed, B cell deficient BALB/c and C57BL/6 mice produced significantly less TGF- $\beta$  and IL-10, respectively three weeks post infection, suggesting that B cells enhance susceptibility to brucella infection through both IL-10 and TGF- $\beta$  dependent mechanisms [37]. The antibody response to *Brucella* spp. is protective but not necessary for immunity [5, 40, 59, 60]. Passive transfer of anti-OPS and anti-outer membrane protein (Omp) antibodies provide protection to mice against brucellosis [17, 61-67]. However, the rough vaccine strain RB51 does not cause the production of anti-OPS antibodies [5, 60]. Furthermore, the antibody-mediated protection against membrane proteins is reduced in smooth strains due to steric hinderance from the OPS of *Brucella* spp. [63]. Taken together, B



cells are capable of both a detrimental or beneficial role in the adaptive immune response against brucellosis.

## **BRUCELLA ABORTUS VACCINES**

It is important to study and refer to the immune responses induced by vaccines known to provide protective immunity in order to develop more effective vaccines [57]. The mouse model is the standard model for brucellosis vaccine and immunity research [17]. The information from this model is not always transferable, but is useful because of the cost and time limitations of using the target species cattle as a model for *B. abortus* vaccine research [17, 68]. The *B. abortus* live-attenuated vaccines Strain 19 and RB51 are the most widely used vaccines in cattle, [69] making them good reference vaccines. The current characteristics of an ideal brucellosis vaccine are: [11, 70].

- Live strain capable of inducing a strong Th1 response
- Does not induce anti-OPS antibodies that interfere with serological tests
- Attenuated and incapable of causing disease or persistence in humans or animals
- A single dose induces long-lasting protection against abortion and systemic or uterine infection
- Does not revert to virulence
- Does not cause seroconversion on revaccination
- Inexpensive and easy to produce and administer

The first vaccine widely used for the control of bovine brucellosis was the live-attenuated *B. abortus* Strain 19 [14], which was originally isolated from the milk of a jersey cow by Dr. John Buck in 1923 [69]. Even though it is effective at controlling brucellosis and inducing protection that lasts the entire productive lifespan of an animal, there are undesirable

characteristics [69]. Some animals remain chronically infected and secrete Strain 19 in milk [69]. As a smooth strain, Strain 19 induces anti-OPS antibodies which make it difficult to distinguish from vaccinated and naturally infected animals [69]. Strain 19 also maintains a low level of virulence, may cause abortion in pregnant cattle, and remains pathogenic to humans [69].

The characteristics of the immune response induced by Strain 19 are a Th1 biased, IFN- $\gamma$  production, antibody production, CD4+ cells, and cytotoxic CD8+ cells in both mice and cattle [57, 69]. Cattle display memory CD4+, CD8+, and B cells on day 210 post vaccination [57, 71]. In cattle, it was determined that CD4+ cells are the primary source of IFN- $\gamma$ , while CD8+ cells didn't produce significant IFN- $\gamma$  [57, 72]. Further, no IL-4 was detected and CD4+ cells were also determined to be the main source of IL-17 [72].

The interference with serological tests and potential for abortion in cattle are the main reasons Strain 19 was replaced with RB51 [69, 73]. RB51 is a stable rough mutant derived from *B. abortus* virulent Strain 2308 passaged on media containing low concentrations of rifampicin and selecting for rough morphology [69, 74]. As an attenuated rough strain, it does not induce anti-OPS antibodies and shows reduced colonization in animals [69, 74, 75]. In addition to cattle, RB51 has been shown to protect water buffalo against *B. abortus* and protects mice against *B. abortus*, *B. melitensis* and *B. ovis* [59, 75]. However, RB51 is also pathogenic to humans, resistant to rifampicin, and not safe for use in pregnant cattle [69].

In both mice and cattle, RB51 induces a strong Th1 response. This includes IFN- $\gamma$  production, CD4+ cells, cytotoxic CD8+ cells, and no detectable IL-4 [57, 69]. Similarly to Strain 19, CD4+ cells are the main source of IFN- $\gamma$  and IL-17 [49, 57, 76]. It should also be noted that IL-17 neutralization in RB51 vaccinated mice significantly reduced protection against

*B. melitensis*, suggesting an important role for the Th17 response in RB51 induced protection [77]. Further, passive transfer experiments revealed that CD8+ cytotoxic activity was positively correlated with IFN- $\gamma$  levels, suggesting that both T cell subsets are important for optimal protection [76].

A key difference between the memory cells generated in cattle by RB51 and Strain 19 is that memory CD4+ and CD8+ cells are induced by both vaccines, but RB51 does not induce memory B cells indicating the humoral response is not important for protection in cattle [57]. CD8+ cells from RB51 vaccinated cattle are granzyme B+ and perforin+, while Strain 19 CD8+ cells are only granzyme B+ [57]. CD8+ cells that secrete granzyme have cytotoxic activity, and cytotoxicity via the perforin pathway has been shown to provide protection against *B. melitensis* in mice [78, 79].

Passive transfer experiments in mice found that T cells from both RB51 and Strain 19 vaccinated mice protected naïve mice from challenge against *B. abortus*, although Strain 19 protection was significantly greater than that conferred by RB51 [59]. Further, serum from RB51 vaccinated mice provided no protection against *B. abortus* while the Strain 19 vaccinated mice serum did [59]. The main difference between the immune responses following Strain 19 and RB51 vaccination of cattle can be generalized by stating that Strain 19 is CD4+ directed while RB51 is CD8+ directed [57]. Further, Th17 and humoral responses appear to enhance RB51 and Strain 19 induced protection, respectively [40, 77].

## **CONCLUDING REMARKS: IMMUNOLOGY**

A vaccine against brucellosis must induce a strong Th1 immune response, including both CD4+ and CD8+ cells, to effectively control infection [5, 17, 35-38]. However, the roles of antibodies, Th2, and Th17 differ between the two commercial *B. abortus* vaccines, indicating

that protection against *Brucella* spp. is best optimized when multiple arms of the immune system are stimulated [17, 40, 49, 57, 61-63]. Indeed, antibody opsonized *Brucella* bacteria are more readily phagocytosized and killed more efficiently in IFN- $\gamma$  activated macrophages; however a small number of bacteria survive and replicate in the macrophage [80]. CD8<sup>+</sup> cells lyse infected macrophages and release the intracellular bacteria to be exposed to another round of opsinization, phagocytosis, and IFN- $\gamma$  enhanced killing [3, 79, 81, 82]. It is important to balance these responses when designing a new vaccine. Differentiation of naïve T cells into Th17 requires stimulation by TGF- $\beta$ , which also downregulates both the Th1 and Th2 differentiation signalling pathways [26]. However, an adaptive Th17 response has been shown to enhance the Th1 mediated protection to brucella infection [77]. Th2 cytokines inhibit IL-12 production and promote antibody production [50, 83]. To achieve maximum protection, a brucellosis vaccine must sufficiently stimulate a Th2 and Th17 response from the immune system without inhibiting the development of a Th1 response.

## **OTHER VACCINATION METHODS**

There are currently no ideal vaccine candidates against brucellosis. As stated previously, the current live-attenuated strains possess safety concerns [69]. Heat-killed *Brucella* bacteria and protein subunit vaccines induce lower protection and a Th2 biased response [84, 85]. The challenge of developing safe brucellosis vaccines capable of stimulating multiple arms of the immune system may be addressed by using multiple design strategies. DNA vaccination has been shown to efficiently stimulate both Th1 and Th2 responses, and including multiple epitopes or epitope dense regions from several antigens induces a broad immune response [15, 85-93]. A DNA vaccine using six nonamer epitopes from different *B. abortus* antigens induced a protective response in the murine model [94]. The overall efficacy may be improved by targeting the

immune response to conserved and relevant antigens [89, 95-98]. Therefore, a multi-epitope DNA vaccine against *B. abortus* may provide increased safety, cross protection, and efficacy compared to the current live-attenuated vaccines.

## **DNA VACCINATION**

DNA vaccination is an immunization method where a DNA plasmid encoding the desired antigen is injected into the host, enters host cells, is then expressed endogenously; and the antigen is secreted from the cell [85, 86]. DNA vaccines are safer than live-attenuated vaccines which have the possibility of reverting to virulence [15, 85]. They are also simpler to manufacture, have lower production costs, are less temperature sensitive, and possess a longer shelf life than most conventional vaccines [15, 85]. A crucial advantage of DNA vaccination is the ability to stimulate a strong cell-mediated response, Th1 [15, 85, 86]. Additionally, the CpG oligodeoxynucleotide (CpG) motifs of bacterial DNA serve as an adjuvant that activates macrophages and dendritic cells, through Toll-like receptor 9 (TLR-9), to secrete IL-12, IFN- $\gamma$  and TNF- $\alpha$  [86, 99]. The expressed antigens are processed and presented via both MHC I and MHC II, which allows both a Th1 and Th2 response to be stimulated [85, 87, 88]. Due to both the safety and the ability to stimulate Th1 immunity, DNA vaccines are seen as a potential alternative to the current live-attenuated vaccines against *Brucella* spp. [15]. DNA vaccination against brucellosis has been well researched and has shown success in the mouse model [15, 87, 88, 94, 100-108]; however, there is no DNA vaccine against brucellosis in large animals.

DNA vaccines are promising but still have disadvantages. This type of immunization is limited to protein antigens, may induce tolerance, and the bacteria or parasite antigens are susceptible to unusual processing [109, 110]. While DNA vaccination in mice has been shown to be effective, low immunogenicity has been a consistent issue in larger animals, including

primates and humans [111]. Further, mice often require multiple high doses that cannot be proportionally applied to large animals [112, 113]. Successful DNA vaccination is also limited by the transfection rate of target cells and expression rate of the antigen once the DNA enters the cell [112, 113]. Unprotected DNA is rapidly degraded in the extracellular space, and DNA must be up taken by the nucleus of host cells to be expressed [112, 113]. Methods to overcome these limitations exist and are being researched to further improve efficacy of DNA vaccination. Electroporation and DNA encoding co-stimulatory molecules targeting APCs has been shown to increase immunogenicity [114]. Further, classical and genetic adjuvants are being researched to enhance immunogenicity [115].

## **MULTI-EPITOPE VACCINES**

Antigen presenting cells present MHC I and MHC II epitopes to CD8<sup>+</sup> and CD4<sup>+</sup> cells respectively [19]. While single epitope vaccinations have been shown to induce protective immune responses, including multiple epitopes and antigens may result in a more complete response and increased protection [27, 67, 89, 116-122]. Multi-epitope vaccines are also able to provide wide population coverage, protect against pathogens with complex life cycles, and induce cross protection [89, 98, 123-125]. It is possible to simultaneously prime responses to multiple specific MHC I epitopes with a single MHC II helper epitope [89]. It has been shown that a single short or long peptide containing intrinsic epitopes with broad MHC binding potential or MHC I and II epitopes specific for multiple MHC alleles is capable of producing a strong immune response in all relevant haplotypes of a population [89, 90]. Immunization with single epitopes, multiple epitopes, and immunodominant regions of antigens has been shown to provide protection against brucellosis in mice [67, 94, 122].

## **EPITOPE INTERACTIONS**

The development of both the specific cytotoxic and antibody response is heavily influenced by CD4<sup>+</sup> cell interactions with CD8<sup>+</sup> cells and B cells, respectively, indicating that protection induced by epitope based vaccines is maximized when MHC I, MHC II, and B cell epitopes are all present [121, 126-129]. Indeed, immunization with MHC II epitopes, also known as CD4<sup>+</sup> ‘helper’ T cell epitopes, has been shown to induce protection against pathogens and enhance the immune response towards MHC I and B cell epitopes and are therefore considered to be crucial in the development of epitope based vaccines [89, 116, 117, 121, 126-130]. Furthermore, it is not necessary for these epitopes to originate from the same antigen or pathogen for CD4<sup>+</sup> dependent enhancement to occur, which allows for the creation of multivalent vaccines [116, 117, 127, 129, 131, 132]. A disadvantage of this reliance of MHC II epitopes is that failure to elicit a sufficient CD4<sup>+</sup> T cell response may result in lower immunogenicity of the vaccine as a whole [133]. The use of promiscuous MHC II epitopes, regions dense with multiple MHC II epitopes, or long peptide sequences containing multiple epitopes has been successful at overcoming this issue [90, 92, 121, 128, 129, 134, 135]. Furthermore, optimal CD4<sup>+</sup> enhancement of B or CD8<sup>+</sup> cell response is achieved when the epitopes are collinearly linked [89, 116, 127-129].

## **CROSS PROTECTION**

On farms where small ruminants and cattle intermingle, *B. melitensis* may be transmitted between the two species; therefore, a vaccine capable of inducing cross protection against multiple *Brucella* spp. is desired [136, 137]. Multi-epitope vaccines induce cross protection by the delivery of several antigens at once or concentrating the immune response on a small number of conserved epitopes rather than polymorphic epitopes [67, 97, 123, 138-141]. DNA/DNA

hybridization studies revealed that members of the genus *Brucella* share over 90% homology [142], and other studies have shown that immunization with immunodominant conserved regions of antigens is effective at inducing cross protection [67].

## ANTIGENS

Antigen	Protection	IFN- $\gamma$	TNF- $\alpha$	Cytotoxic CD8+	Other Information	Source
Cu/Zn SOD	1.15 - 2.16	+	-	+	Known protective epitope	[87, 88, 103, 108, 143-147]
BP 26	1.6 - 2.6	+	NA	NA	Protects bison, immunogenic in sheep	[56, 148, 149]
Omp19	1.38 - 1.85	+	NA	NA	Mucosal protection, activates DC, induces IL-17, protective antibodies	[39, 53, 54, 67]
Omp16	1.15 - 1.97	+	NA	NA	Activates DC, mucosal protection, induces IL-17, protective antibodies	[39, 41, 104]
Omp25	1.39 - 2.7	+	+	+	Antibodies are protective	[64, 101, 150, 151]
L9	1.96 - 2.55	+	+	NA	Protection is CD8+ mediated	[15, 152]
L7/L12	0.84 - 1.79	+	+	+		[105, 153-155]

**Table 1.1:** Antigen Characteristics

Table of antigens included in the DNA vaccines. Systemic protection induced by the antigen against *B. abortus* or *B. melitensis* was determined by log CFU reduction recovered from spleen of challenged mice determined at 7-30 days post infection. Characteristics of the immune response induced by vaccination with the antigen of interest, including IFN- $\gamma$  and TNF- $\alpha$  production and induction of specific CD8+ cytotoxic activity. Sources indicate whether the antigen produces (+), does not produce (-), or if no data was found (NA) with regards to the characteristic of the immune response.

## CU/ZN SUPEROXIDE DISMUTASE

The *Brucella* spp. express an immunogenic 18.5-20 kDa periplasmic Cu/Zn superoxide dismutase (SOD) [87, 108, 147], a class of enzymes which allow bacteria to survive the respiratory burst of phagocytes [156, 157]. *B. abortus* SOD deletion mutants display reduced



survival during early infection *in vivo* [158]. Strain 19 and RB51 vaccinated cattle and mice do not produce a humoral or cell-mediated immune response against SOD [122, 143], indicating that SOD is not a necessary antigen for a protective immune response [122]. However, immunization with SOD has been found to consistently grant protection against brucellosis when administered in the form of either recombinant peptides, DNA vaccines, whole recombinant proteins, overexpression mutants, or through co-immunization with other antigens [87, 88, 100, 103, 107, 108, 146, 147].

Mutant strains of RB51 overexpressing SOD (RB51SOD) provided greater protection than the parental strain in mice, without increased survival or persistence [143, 145, 146]. Only the RB51SOD vaccinated group produced IFN- $\gamma$  when splenocytes were stimulated with rSOD [143]. IgG2a was the only detected antibody isotype against Cu/Zn SOD in RB51SOD vaccinated mice [143]. Taken together, this indicates that SOD produces a Th1 response when overexpressed in RB51 vaccinated mice [143]. Another RB51-SOD overexpression mutant provided significantly greater protection against *B. suis* in mice than parental RB51 at two weeks post challenge, indicating SOD is a cross protective antigen [145].

In mice, DNA vaccinations encoding SOD have consistently induced protection at two to four weeks following challenge and display a Th1 biased immune response characterized by IFN- $\gamma$  producing CD4<sup>+</sup> cells, specific CD8<sup>+</sup> cytotoxic activity, splenocyte proliferation, and low or no detectable IL-10 and IL-4 [87, 88, 103, 108]. Depending on route of immunization and dosage, both CD4<sup>+</sup> and CD8<sup>+</sup> have been observed to be the primary source of IFN- $\gamma$  [87, 103]. Nasal vaccination with SOD-DNA produces both IgG2a and IgA antibodies as well as CD8<sup>+</sup> splenocytes and systemic protection against *B. abortus* in mice [108]. SOD DNA vaccinated calves showed significant IgG1 and IgG2a titers and IFN- $\gamma$ <sup>+</sup> peripheral blood monocytes

(PBMC) but no detectable TNF- $\alpha$  or IL-4 on stimulation with rSOD or crude brucella proteins [144].

Mice vaccinated with the synthetic SOD peptide GGAPGEKDGKIVPAG, with or without adjuvant, showed significant protection two weeks following *B. abortus* challenge [147]. The GGAPGEKDGKIVPAG peptide vaccinated mice produced significant amounts SOD specific IgG antibodies [147]. Pratt et al. hypothesized that antibody binding to the corresponding epitope in the whole protein may inhibit SOD function therefore the bacteria will be more susceptible to the respiratory burst [157]. To our knowledge this has not been tested. This peptide is not recognized by the humoral or cell-mediated immune response of cattle vaccinated with Strain 19 or RB51 [122].

## **BP26**

BP26, also known as Omp28 [152], is a 26 kDa periplasmic protein of unknown function that is immunogenic and immunodominant in cattle, dogs, goats, sheep, and humans [60, 159]. BP26 is highly conserved between *B. melitensis*, *B. abortus*, *B. ovis*, *B. suis* and *B. neotomae* [148, 149, 160]. A Strain 19 mutant with the BP26 gene deleted (S19 $\Delta$ BP26) maintained the same residual virulence and protection as parental Strain 19 in BALB/c mice and cattle [60, 159], indicating that BP26 is not necessary for protective immunity to brucellosis [60]. However, BP26 is capable of inducing both humoral and cell-mediated responses and is protective as a subunit or DNA vaccine [56, 149, 160, 161].

Immunization with recombinant BP26 in BALB/c mice resulted in approximately 2.6 logs of protection against *B. abortus* 10 days post-infection [148]. DNA vaccination with BP26 resulted in 1.16 log less CFU in the spleen of BALB/c mice when challenged with *B. melitensis* [149]. Twelve epitopes of BP26 are capable of inducing IFN- $\gamma$  secretion from PBMCs of sheep

between 1 to 7.5 months after vaccination with *B. melitensis* M5-90 [160]. BP26 DNA vaccination is capable of producing IFN- $\gamma$  and both specific CD4<sup>+</sup> and CD8<sup>+</sup> cells in sheep and bison [56, 160], suggesting this antigen is a candidate for immunization in multiple animal and *Brucella* species.

## **OMP19**

Omp19 is an 18-19 kDa outer membrane surface exposed lipoprotein that is associated with virulence and present in all biovars of the six classical *Brucella* species [39, 54, 60, 159]. Strain 19 and virulent *B. abortus* Omp19 deletion mutants are attenuated in mice, do not confer protection against *B. abortus* challenge in heifers, and display reduced growth in solid media [60, 159, 162]. Further, CD4<sup>+</sup>, CD8<sup>+</sup> and antibodies specific to Omp19 are capable of inducing systemic and mucosal protection against brucellosis [39, 67].

Mice immunized with rOmp19 or an immunodominant region of Omp19 (amino acids 21 – 177) display systemic protection against both *B. melitensis* and *B. abortus* when challenged [39, 53, 67]. Monoclonal antibodies against Omp19 protect mice from challenge with the rough strain *B. ovis* [39] and bind to heat-killed rough strain RB51 but not smooth strain *B. abortus* 544. This suggests steric hindrance due to OPS interference with antibodies binding to the surface exposed Omp19 motifs [39]. However, sera from mice immunized with rOmp19<sub>21-177</sub> protected macrophages from cytopathic effects of both *B. abortus* and *B. melitensis in vitro* [67], suggesting that concentrating the antibody response on this region is an effective immunization strategy.

Splenocytes from both whole rOmp19 and rOmp19<sub>21-177</sub> vaccinated mice produce IFN- $\gamma$  and IL-12 but not Th2 cytokines on stimulation [39, 53, 67]. The IFN- $\gamma$  producing cells were determined to be CD4<sup>+</sup> and CD8<sup>+</sup> cells; and depletion of either subset reduced, but did not

completely eliminate, protection against challenge [39]. Recombinant Omp19 has also been shown to activate dendritic cells and provide mucosal protection against *B. abortus* [39, 54]. Intragastric (i.g.) immunization of mice with rOmp19 has been shown to induce a Th1 and Th17 response while intraperitoneal (i.p.) vaccination only induced a Th1 response [39, 53, 54]. This oral protection is Th17 dependent [54].

## **OMP16**

Omp16 is a 16.5 kDa outer membrane surface exposed lipoprotein expressed in all biovars of the classical *Brucella* species [39, 104]. No successful Omp16 deletion mutant has been produced in any of the *Brucella* spp. [163], suggesting that this protein is necessary for survival. Omp16 acts as an adjuvant by activating the CD8 $\alpha$ <sup>+</sup> subset of dendritic cells both *in vivo* and *in vitro* in C57BL/6 mice [41]. This activation is dependent on TLR-4 *in vivo* but not *in vitro* [41].

Vaccination with both Omp16-DNA and rOmp16 induces specific IFN- $\gamma$ <sup>+</sup> splenocytes, antibodies, and no detectable Th2 cytokines [39, 41, 104]. Monoclonal antibodies against Omp16 have been shown to provide significant protection in mice against *B. ovis* challenge [39, 104]. In rOmp16 vaccination, antigen specific CD4<sup>+</sup> and CD8<sup>+</sup> cells were found to contribute to the production of IFN- $\gamma$ ; and depletion experiments revealed that both subsets provide significant protection, but the protection is greatest when both populations are present [39]. Omp16 with cholera toxin and i.g. immunization of mice provided significant protection at four weeks post oral challenge with *B. abortus* similar to RB51 [39, 41]. In summary, vaccination with Omp16 induces a strong Th1, humoral response, and mucosal protection against multiple *Brucella* spp. [39, 41, 104].

## OMP25

Omp25 is an immunogenic, surface exposed, 25 kDa transmembrane protein that is highly conserved [64, 150, 164]. Immunization with Omp25 inconsistently results in TNF- $\alpha$  production which is contradictory to evidence that Omp25 of *B. suis* inhibits TNF- $\alpha$  production in human macrophages and allows secretion of periplasmic proteins in acidic medium [64, 101, 150, 151, 165]. Deletion of Omp25 in *Brucella* spp. results in lower persistence within the animal and reveals Omp25 is not necessary for protection against smooth and rough *Brucella* spp. in mice or goats [164, 166, 167]. Omp25 is also known to bind the outer membrane to the peptidoglycan layer [150, 164], which suggests a role in membrane stability, persistence, TNF- $\alpha$  inhibition, and adaptation to acidic environment. While deletion mutants indicate that Omp25 is not necessary for protection against *Brucella* spp., it is still a known protective antigen against *B. abortus* and *B. melitensis* [101, 151].

In sheep, as many as 91% of *B. melitensis* infected sera contain antibodies to Omp25 [101], indicating that Omp25 is both immunodominant and broadly recognized. Further, antibodies against Omp25 provide limited but significant protection against *B. melitensis* [64], which suggests that a vaccine incorporating Omp25 may induce protective antibodies in a large percentage of the population.

Mice vaccinated with plasmid DNA encoding Omp25 displayed significant protection against *B. melitensis* 15 days post challenge, and no bacteria were recovered from three of five mice [101]. Splenocytes from mice vaccinated with Omp25 coding plasmid DNA displayed significant production of IFN- $\gamma$  and TNF- $\alpha$ , but not IL-4 or IL-10, on stimulation with rOmp25 at weeks 5, 8, 11 and 21 following initial vaccination [101], indicating that a memory Th1 immune response was induced. Splenocytes from mice vaccinated with rOmp25 produced significant IL-

12, IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-6 on stimulation with rOmp25 [150]. Recombinant Omp25 also induces in both CD4+ and CD8+ cells cytotoxic killing of infected macrophages [151]. In conclusion, Omp25 displays several desirable features for a brucellosis vaccine such as the induction of protective antibodies and a strong Th1 response, including IFN- $\gamma$ , TNF- $\alpha$  and specific cytotoxic CD8+ cells [64, 101, 150, 151].

## **RIBOSOMAL L9**

L9 is a conserved 50S ribosomal protein in *Brucella* spp. [152]. Vaccination with recombinant or DNA L9 produces IgG isotype one week post vaccination [15, 152]. When stimulated with rL9, splenocytes from L9 DNA vaccinated mice produced significant quantities of both IFN- $\gamma$  and TNF- $\alpha$ . However, IL-2 production was only significant when the DNA vaccine was introduced through electroporation; and IL-4 was only detected in rL9 + Alum vaccinated mice [15, 152]. Overall, the IFN- $\gamma$  producing CD8+ cell population was significantly larger than the CD4+ population in all groups; and electroporated DNA vaccination induced the highest CD8+ counts [15]. All groups displayed significant protection against *B. abortus* 30 days post challenge; however only the electroporated group was comparable to S19 [15, 152]. Together, the data suggests that L9 DNA vaccination induces CD8+ mediated protection against *B. abortus*.

## **RIBOSOMAL L7/L12**

The brucella 50S ribosomal subunit L7/L12 is an immunodominant Th1 antigen in both cattle and mice [153, 168]. Splenocytes from mice vaccinated with Strain 19 proliferate in response to rL7/L12 but do not produce anti-L7/L12 antibodies [153]. CD4+ PBMCs from cattle vaccinated with rL7/L12 proliferate in response to recombinant L7/L12 [168], indicating that a cell-mediated response to this antigen is not species restricted. Without adjuvant, rL7/L12 failed

to provide protection against *B. abortus* challenge while rL7/L12 in various adjuvants or L7/L12 DNA vaccination has been shown to provide protection 7 to 30 days post challenge [104, 153-155]. Specific cytotoxic CD8<sup>+</sup> cells were present in Escheriosome rL7/L12 vaccinated mice, but no CD4<sup>+</sup> cytotoxic activity was detected [153]. Splenocytes from recombinant and DNA vaccinated L7/L12 mice produced significant IFN- $\gamma$  and TNF- $\alpha$  and low but significant amounts of IL-4 when stimulated with rL7/L12 [104, 153-155].

## COMBINATION VACCINATION

Immunization with multiple antigens has been shown to effectively enhance protection against brucellosis. Co-administration of both recombinant and DNA vaccines of *B. abortus* BP26 and trigger factor showed greater protection than either did individually [149, 161]. It is also possible that co-immunization does not increase protection. In mice, rOmp16 + rOmp19 immunization did not significantly alter protection compared to the individual recombinant proteins against *B. abortus* challenge [41]. However, immunization with recombinant immunodominant regions of Omp19 and P39 enhances antibody production and protection against both *B. abortus* and *B. melitensis* compared to immunization with either individual antigen [67]. Furthermore, Omp16 co-immunized with other antigens enhances protection [104, 169], suggesting that the proper combination of antigens is more important than simply including more antigens.

A divalent DNA vaccine encoding both L7/L12 and Omp16 induces significantly higher antibody titers, IFN- $\gamma$ <sup>+</sup> splenocytes, and protection against *B. abortus* than either univalent DNA vaccine [104]. Another study found an influenza viral vector expressing L7/L12 and Omp16 provided long term protection against *B. abortus* and *B. melitensis* in both pregnant and non-pregnant cattle [169-172]. The immune response consisted of IgG2a dominate isotype, CD4<sup>+</sup>

and CD8<sup>+</sup> cells, and IFN- $\gamma$  production [169-172]. This vaccine was found to provide protection against abortion and heifer and fetal colonization comparable to Strain 19 [170-172]. These studies indicate that vaccination with the combination of L7/L12 and Omp16 is an effective way of increasing protection against *B. abortus* and *B. melitensis* [104, 171].

A combination DNA vaccine of BCSP31, SOD and L7/L12 provided significantly greater protection against *B. abortus* than the univalent DNA vaccines did in mice [100]. IFN- $\gamma$  producing CD4<sup>+</sup> and CD8<sup>+</sup> cells specific to each protein were detected [100]. Protection was mediated primarily by CD4<sup>+</sup> cells but was highest when both subsets were present [100]. Further, both subsets were capable of specific cytotoxic activity, but CD8<sup>+</sup> cells displayed higher activity [100].

Mice co-immunized with recombinant immunodominant regions of Omp19 + P39 were significantly more protected from both *B. abortus* and *B. melitensis* than the univalent groups [67]. Sera from rOmp19 + rP39 vaccinated mice protected macrophages from cytopathic effects of *B. abortus* and *B. melitensis in vitro* significantly more than either recombinant antigen individually [67]. Splenocytes from the rOmp19 + rP39 group produced significantly higher levels of IFN- $\gamma$ , IL-2, and IL-12 than the univalent groups; and IL-4 was not detected in any group. Interestingly, the P39 group did not produce detectable IL-12 while the rOmp19 + rP39 produced significantly more than the rOmp19 group. This data suggests an advantage to using multiple antigens may result in a synergistic effect which induces cytokines an antigen normally would not stimulate and increases overall stimulation of the immune system [67]. In conclusion, this study indicates that co-immunization with regions of antigens is capable of increasing both protection and cross protection against *Brucella* species.



The *Brucella* antigens Cu/Zn SOD, Omp19, Omp16 and L7/L12 have been shown to induce a strong Th1 response, cross protection and provide higher protection when co-immunized other antigens [67, 100, 104, 145, 171]. Immunization with these antigens has also revealed other desirable characteristics such as mucosal immunity [39, 54], cytotoxic CD8+ activity [100, 153], protective antibodies [39, 67, 104], and activation of APCs [41]. The antigens BP26, L9, and Omp25 have not been extensively used in co-immunization studies but reliably provide high levels of protection [15, 64, 148-152]. Aside from inducing protection and a strong Th1 response, these antigens are conserved [148, 149, 160, 164], recognized in multiple animal species [56, 60, 101, 159, 160], induce TNF- $\alpha$  [15, 101, 150], and cytotoxic CD8+ activity [151].

## **HYPOTHESES AND SPECIFIC AIMS**

The overall goal of this project is to design a nonliving vaccine capable of inducing protection against *B. abortus* infection. To achieve this, two DNA vaccines p425/BabV1 and p425/BabV2 were constructed using bioinformatics technology. The hypothesis for this work is that it is possible to design a DNA vaccine recognized by the humoral immune response of *Brucella abortus* Strain 19 vaccinated goats. To test this hypothesis, the following aims were pursued.

1. The p425/BabV1 and p425/BabV2 were designed and constructed from protective epitopes.
2. The p425/BabV1 and p425/BabV2 can be transfected into eukaryotic cells.
3. The *BabV1* and *BabV2* genes are transcribed to RNA.
4. The novel BabV1 and BabV2 proteins may be recognized by sera from Strain 19 vaccinated goats.

## CHAPTER II MATERIAL AND METHODS

### ANTIGEN AND EPITOPE SELECTION

Literature mining was performed to search for known protective antigens and epitopes from *B. abortus*. Sequences for the antigens were obtained from NCBI Genbank [173] (Table 2.1). MHC II epitope selection was performed using 15mer binding data from Epimatrix [174] and IEDB-II [175]. MHC I data was obtained using IEDB-I and Epimatrix to search for nonamers [175]. The human and bovine MHC alleles used are listed in Table 2.2. RANKPep was used to predict proper proteasome processing [176]. Overlapping regions of MHC I and MHC II binding epitopes and known protective epitopes were selected to be included in the plasmids. Selected epitopes are listed in Table 2.3.

	Accession Number
SOD	WP_002972093.1
Omp25	EFH34586.1
BP26	WP_006229497.1
Omp19	EEP62573.1
Omp16	WP_002966947.1
L7/L12	WP_002964371.1
L9	WP_002963608.1

**Table 2.1** Protective *B. abortus* Antigen Accession Numbers  
Table of *B. abortus* protective antigens and the corresponding NCBI Genbank accession number.

### PLASMID CONSTRUCTION

The *BabV1* and *BabV2* conalbumin signal sequence and open reading frames were codon optimized for *Criteculus griseus* and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The *BabV* genes were under the control of CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-899, CTC, 900-918) and contain the conalbumin signal sequence (Genbank X02009 base pairs (bp) 74-133) [173]. The *BabV1* open reading frame contains

epitopes from SOD, L7/L12, Omp16, and Omp19 with free rotational spacers between epitopes. The *BabV2* open reading frame contains epitopes from SOD, L9, BP26, and Omp25 with free rotational spacers between epitopes. The free rotational spacers allow the protein to fold in a way that would produce conformational epitopes similar to the native antigen [177]. The vector p425 is a heavily modified pBluescript vector and was obtained from laboratory stocks. Plasmid p425 contains the chicken ovalbumin polyA tail sequence (GenBank Accession #J00895 base pairs 8260-9175) and a Puromycin resistance gene (pMOD PURO (InvivoGen) bp 717-116).

Human MHC I Alleles	Bovine MHC I Alleles	MHC II Alleles
A0101	BoLA-1*01901	DRB1*0101
A0201	BoLA-2*01201	DRB1*0301
A0301	BoLA-4*02401	DRB1*0401
A2402	Bola-6*01301	DRB1*0701
B0702	BoLa-3*01701	DRB1*0801
B4403	BoLa-2*00801	DRB1*1101
	BoLa-2*1601	DRB1*1301
	BoLa-1*2301	DRB1*1501

**Table 2.2** Selected MHC Alleles

Table of the human MHC I and MHC II alleles and bovine MHC I alleles used to predict immunogenic epitopes via IEDB I, IEDB II, and Epimatrix.

The pBabV1, pBabV2, and p425 plasmids were digested with Anza restriction enzymes BshTI and SalI (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Digested DNA bands were extracted from a 1% agarose SYBR®Safe gel (Thermo Fisher Scientific, Waltham, MA) using a razor. Agarose was dissolved and the digested DNA was concentrated using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA) according to the manufacturer's directions. Digested conalbumin signal sequence and open reading frames of *BabV1* and *BabV2* were ligated into the digested p425 using Quick Ligase™ (New England

Biolabs, Ipswich, MA) according to kit's recommendation to make p425/BabV1 and p425/BabV2, respectively.

Antigen	Amino Acid Position
SOD	50-58
SOD	75-86
SOD	125-149
L7/L12	14-42
L7/L12	52-96
Omp16	70-99
Omp16	103-137
Omp19	79-87
Omp19	132-163
L9	19-51
L9	108-125
BP26	51-79
BP26	166-187
BP26	230-250
Omp25	112-138
Omp25	53-75

**Table 2.3** List of *B. abortus* Epitopes Used in DNA Vaccine Construction

Table of antigens and corresponding amino acid residues predicted to be immunogenic epitopes by IEDB-I, IEDB-II, Epimatrix, and RANKPep which are included in the p425/BabV1 and p425/BabV2 vaccines.

Sequences were PCR amplified using the primer sets in Table 3.4 and AccuPrime™ DNA polymerase enzyme and buffer (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Parameters for PCR were 95°C for 4 minutes then 25 cycles of 95°C for 30 seconds, annealing temperature (Table 2.4) for 30 seconds, 68°C for 45 seconds, and a final extension segment of 68°C for 5 minutes was performed. Gel electrophoresis was used to determine the size of the plasmids or PCR products. Briefly, 1% agarose gels containing ethidium bromide were electrophoresed at 90 mA and UV transillumination was used to visualize the bands. The ladders used were supercoiled ladder (Bayou Biolabs, Metairie, LA) for

undigested plasmid samples, 1 Kbp (Bayou Biolabs, Metairie, LA) and 100 bp ladders (Bayou Biolabs, Metairie, LA) for digested plasmids, and 100 bp ladder for PCR products. The products were sequenced by GeneLab in the School of Veterinary Medicine at Louisiana State University using the Sanger dideoxynucleotide sequencing method.

Primer Name	Sequence 5' to 3'	Product Length (bp)	Annealing Temperature (°C)
BabVpolyA F	TAT GGG GGA AAA ATG CAG CCT	756	53
BabVpolyA R	CAA TAC GCC CGC GTT TCT T		
BabV1 F	CAA GCT CAA TGG CTC CAA CG	847	51.5
BabV1 R	AGC AAT TGC CTT GTC AGC AT		
Conss1 F	CGT CGT CGA CAA CAT GAA GC	804	54.8
Conss1 R	GCC CTT CTT TGA CC AAT GC		
BabV2 F	CTA TGA CCG CGA ACA ACG ACG AG	843	51.5
BabV2 R	CTG GAG CAG CTT GGT CAT ACA		
Conss2 F	CGT CGT CGA CAA CAT GAA GC	745	53
Conss2 R	GTT CGC GAC TGC TCG TTT G		

**Table 2.4** BabV Primer Sets

Table containing the primer sets used to PCR amplify and sequence the *BabV1* and *BabV2* genes in p425/BabV1 and p425/BabV2. The primer sets were designed to overlap in a way that allowed the entire *BabV1* and *BabV2* genes to be accurately sequenced. Sequence of the forward and reverse primers, predicted product size in base pairs, and annealing temperature are included.

## CELLS AND TRANSFECTION

Plasmids were maintained and replicated in MAX Efficiency® Stbl2™ competent bacteria (Invitrogen, Carlsbad, CA) incubated at 37°C. *Escherichia coli* were transfected with pBabV1, pBabV2, p425/BabV1, and p425/BabV2 plasmids using the manufacturer's directions. Ampicillin was used to select for transfected cells. Plasmids were harvested using ZymoPURE™ Plasmid Midiprep (Zymo Research, Carlsbad, CA) or Zyppy™ Plasmid Miniprep (Zymo Research, Carlsbad, CA) kits according to the kit's recommendations.

Recombinant proteins were expressed in a Chinese hamster ovary (CHO K1) (ATCC, Manassas, VA) cell line cultured in Corning® SF media (Corning Inc., Corning, NY) supplemented with 5% fetal bovine serum, glutamax (Gibco, Grand Island, NY), and incubated at 37°C with 5% CO<sub>2</sub> and humidity. The CHO K1 cells were grown to confluence in T150 flasks then washed with room temperature PBS (Gibco, Grand Island, NY) then passaged using TrypLE™ Express (Gibco, Grand Island, NY) to disassociate and split cells according to the manufacturer's directions into six well plates. CHO K1 cells were transfected 24 hours after passaging with Lipofectamine™ 3000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. CHO K1 cells transfected with p425/BabV1 and p425/BabV2 are referred to as CHOB1 and CHOB2, respectively. Transfected CHO K1 cells were selected using media supplemented with 50 µg/ml puromycin 24 hours to allow cells to recover. One week after transfection, individual clusters were picked with a sterile inoculating loop and transferred to individual wells. When confluent in the wells, the cloned cells were disassociated with TrypLE™ Express and transferred to a T25 flask.

When CHOB1, CHOB2, and negative control CHO K1 cells were confluent, the T25 flasks were washed twice with PBS and cultured with Corning® SF media (Corning Inc., Corning, NY) supplemented with glutamax (Gibco, Grand Island, NY) and 50 µg/ml Puromycin (absent in negative control media) and incubated at 37°C with 5% CO<sub>2</sub> and humidity. After 72 hours, media was collected and cells were washed twice with PBS. Cells were then disassociated with TrypLE™ Express and collected in a 15 ml tube. Serum free media and cell samples were stored at -20°C until testing.

## GENOMIC DNA EXTRACTION AND RT-PCR

Genomic DNA was extracted from the CHOB1, CHOB2, and negative control CHO K1 cells using a DNEasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The *BabV1* and *BabV2* genes were PCR amplified using the primer sets listed below (Table 2.5). Parameters for PCR were 95°C for 4 minutes then 30 cycles of 95°C for 30 seconds, annealing temperature (Table 2.5) for 30 seconds, and 68°C for 25 seconds followed by a final extension of 68°C for 5 minutes. The size of PCR products was determined via gel electrophoresis procedure as described above. Products were sequenced by GeneLab in the School of Veterinary Medicine at Louisiana State University using the Sanger dideoxynucleotide sequencing method.

To determine if the *BabV1* and *BabV2* genes were transcribed, RNA extraction was performed using an RNEasy® Plus Micro Kit (QIAGEN, Hilden, Germany) according to the kit's recommendations. The RNA sample was immediately used as a template with an OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) using the primers in Table 2.5. The thermocycler parameters for reverse transcription and initial PCR activation were 50°C for 30 minutes then 95°C for 15 minutes, respectively. This was followed by 40 cycles of 94°C for 30 seconds, annealing temperature (Table 3.5) for 30 seconds, then 72°C for 1 minute and a final extension of 72°C for 10 minutes. The PCR fragments were gel extracted using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA) and sequenced by GeneLab in the School of Veterinary Medicine at Louisiana State University using the Sanger dideoxynucleotide sequencing method.

Primer Name	Sequence 5' to 3'	Annealing Temperature (°C)	Product Size (bp)
CHO GADPH F	CGA GAT CCC GCC AAC ATC AA	54	501
CHO GADPH R	TGT CAG ATC CAC AAC GGA CAC	54	
B1Conss F	ACC GTC GTC GAC AAC ATG AAG	55	694
B1Conss R	ACT GGG GAT ATC GTT GGA GC	55	
B1BabV F	TTC GAT GTT GTC CTT GCC GA	54	920
B1BabV R	GAA GGC TGC ATT TTT CCC C	54	
B2Conss F	GTC GTC GAC AAC ATG AAG CTC	53	636
B2Conss R	CTC GTT GTT CGC GGT CAT AG	53	
B2BabV-1 F	AAA CTA TTG GAC TCG GCC CT	54	485
B2BabV-1 R	TCC TGC AGT CAA GTA CGG C	54	
B2BabV-2 F	CCT GGT GGG AGT CCT GAC AT	53	730
B2BabV-2 R	GAA TTG CAC CTG CTG TTC TCA CAG T	53	
BCHOpolyA-1 F	GTC CCT TCC AAC AAG ACC CA	54	676
BCHOpolyA-1 R	TCC CTG CTA CAC AAC CTT GC	54	
BCHOpolyA-2 F	CCA TTC CTA TGC TGA CAA GGC	54	688
BCHOpolyA-2 R	CTG GCA CTC TGT TAC CC	54	

**Table 2.5** CHOB Primer Sets

Table of primer sets used during PCR, RT-PCR, and sequencing *BabV1* and *BabV2* from genomic DNA and RNA including the 5' to 3' sequence of the forward and reverse primers. The CHO GADPH primer set is specific to a housekeeping gene in CHO K1 cells and used as a positive control. The primer sets were designed to overlap in a way that allowed the entire *BabV1* and *BabV2* genes to be accurately sequenced. The predicted product size in base pairs and annealing temperature used in the PCR and RT-PCR protocols are included.

## SERA AND BRUCELLA ANTIGENS

Sera from *B. abortus* Strain 19 vaccinated goats was pooled from laboratory stocks collected from experimentally infected animals (see Appendix B) and diluted 1:7500 in 1% gelatin solution (TBS/Tween 20%) to make the primary antisera for use in Western blots. The secondary antibody donkey anti-goat (Abcam, Cambridge, UK) was diluted 1:5000 in 1% gelatin solution (TBS/Tween 20%). Acetone-killed *B. abortus* Strain 2308 was acquired from legacy stocks made prior to 1995 and suspended in water at a concentration of 1 mg/ml.



## WESTERN BLOT

Cell samples were thawed and pelleted by centrifugation at 500g for 10 minutes and supernatant was then discarded. Cells were resuspended in 5 ml of PBS, and cell counts were normalized using Volupack tubes (Techno Plastic Products, Trasadingen, Switzerland). Equal numbers of cells were lysed with M-Per™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's recommendations. Serum-free media and lysate samples were stored at -20°C until testing.

To prepare samples for Western blot, 15 µl of 2x Laemmli Sample Buffer (Sigma Aldrich, St. Louis, MO), 10 µl of 10x DTT and 15 µl of serum free media, cell lysate samples, or *B. abortus* Strain 2308 antigen were mixed then heated to 100°C for 10 minutes. The standard used was Precision Plus Protein™ Dual Color Standards 10-250 kDa (Bio-Rad, Hercules, CA). Samples were run at 70V for 10 minutes then 200V for 80 minutes in a Criterion™ Precast Gel (Bio-Rad, Hercules, CA). After electrophoresis was completed, the gel was placed in transfer buffer (20% methanol in Tris-glycine electrode buffer) for 5 minutes to equilibrate.

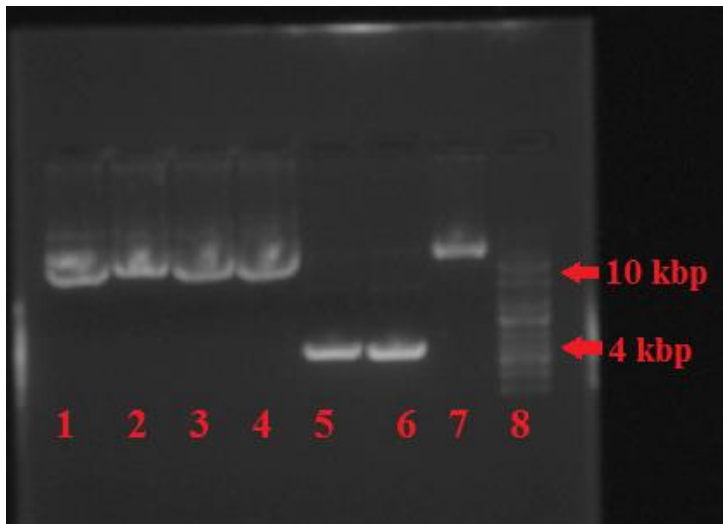
Nitrocellulose was rehydrated in transfer buffer for 30 minutes. Electrophoretic transfer to the nitrocellulose was performed at 25V for 50 minutes. The nitrocellulose membrane was rinsed with TBS/Tween 20% and the membrane was blocked in TBS/Tween 20% with 5% milk solution for 1 hour at 37°C in a shaking incubator. The membrane was washed four times with TBS/Tween 20% for five minutes each wash. Membranes were incubated with the primary antisera for one hour at room temperature with shaking and then rinsed in TBS/Tween 20% three times for five minutes per wash. Donkey anti-goat secondary antibody diluted to 1:5000 in 1% gelatin TBS/Tween 20% was incubated with the membrane at room temperature for one hour while shaking. The membrane was rinsed and washed with TBS/Tween 20% four times with

five minutes per wash then rinsed with distilled water. The color reaction of the membrane was developed in 1-Step Ultra TMB-Blotting Solution (Thermo Fisher Scientific, Waltham, MA) until bands appeared, then washed with distilled water and air dried.

## CHAPTER III RESULTS

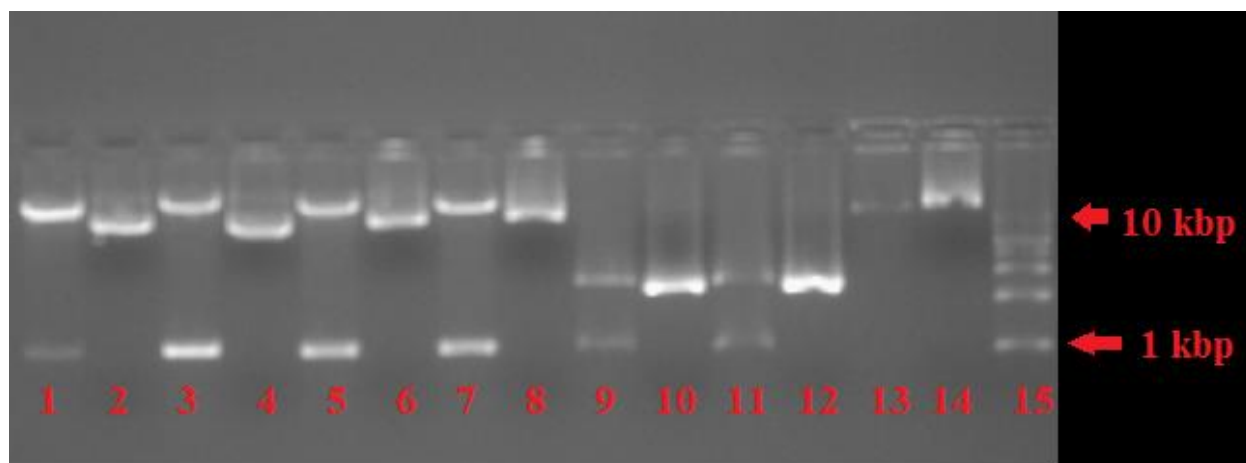
### PLASMID CONSTRUCTION AND CHO K1 TRANSFECTION

In order to confirm the plasmids were constructed properly, gel electrophoresis was used to determine if the plasmids, digested DNA, and PCR products matched the expected sizes. The harvested p425, pBabV1, pBabV2, p425/BabV1, and p425/BabV2 plasmids matched the expected sizes on gel electrophoresis (Figure 3.1). The products of BshTI and SalI digestion of the p425, pBabV1, and pBabV2 plasmids were the expected sizes, as well (Figure 3.2). BabV1/425 and BabV2/425 plasmids were PCR amplified, and the products matched the predicted sizes (Figure 3.3). The PCR products were sequenced to confirm the correct sequence. Puromycin successfully selected for transfected CHOB1 and CHOB2 cells. Approximately five colonies of CHOB1 and CHOB2 cells per well survived after introduction of 50 µg/ml puromycin to the media. Replicating CHO K1 cells were observed five days post introduction of puromycin, and no replicating CHO K1 cells were observed in the untreated control wells.



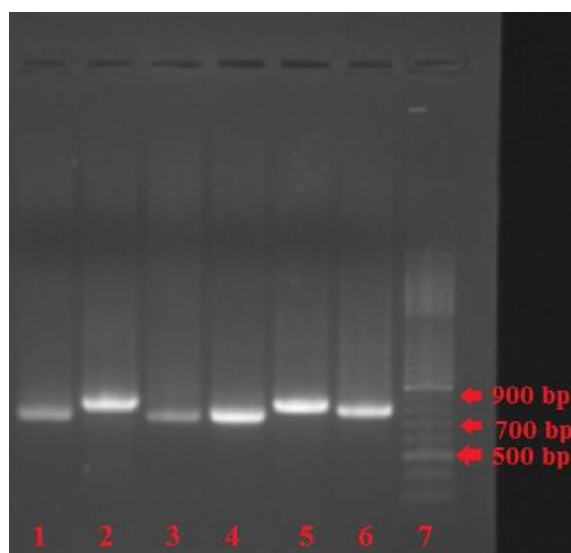
**Figure 3.1.** BabV Plasmid Gel Electrophoresis

A gel electrophoresis was run on the original 7.) p425 (14,977 bp), 6.) pBabV1 (3,855 bp), and 5.) pBabV2 (3,862 bp). 8.) supercoiled ladder. The p425 with the *BabV1* and *BabV2* inserts are referred to as 3-4.) p425/BabV1 (15,277 bp) and 1-2.) p425/BabV2 (15,250 bp) respectively.



**Figure 3.2.** BabV SalI and BstHI Digested Samples

A gel electrophoresis was run on the undigested 10.) pBabV2, 12.) pBabV1, and 14.) p425 and their digestion products with a 15.) 1 Kbp ladder. The restriction enzymes SalI and BstHI were used to extract the *BabV1* and *BabV2* inserts from the plasmids and prepare p425 for ligation. The 11.) *BabV1* (1,076 bp) or 9.) *BabV2* (1,103 bp) inserts were ligated into digested 13.) p425 (14,179 bp) to make 6 and 8.) p425/*BabV1* and 2 and 4.) p425/*BabV1*, respectively. The completed 5 and 7.) p425/*BabV1* (14,179 and 1,076 bp bands) and 1 and 3.) p425/*BabV2* (14,179 and 1,103 bp bands) were digested again to confirm the inserts were present.

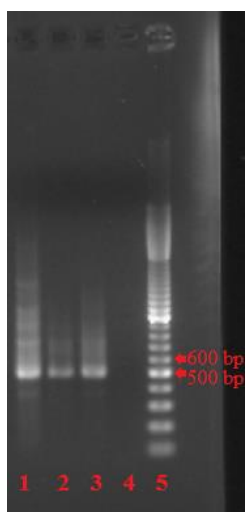


**Figure 3.3.** BabV PCR Gel Electrophoresis

A gel electrophoresis was run on PCR products with a 7.) 100 bp ladder. The entire *BabV1* and *BabV2* were PCR amplified from p425/*BabV1* and p425/*BabV2* respectively with three primer sets per plasmid. These products were sequenced to confirm the expected sequence. The products of PCR with template p425/*BabV2* and primer set 1.) BabVpolyA (756 bp), 2.) BabV2 (843 bp), and 3.) Conss2 (745 bp). The products of PCR template p425/*BabV1* and primer set 4.) BabVpolyA (756 bp), 5.) BabV1 (847 bp), and 6.) Conss1 (804 bp).

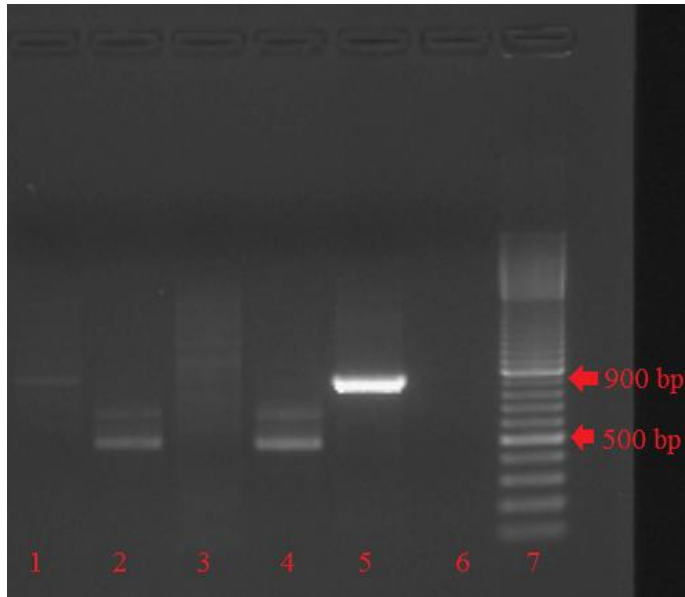
## GENOMIC DNA PCR AND SEQUENCING

The gCHOB1, gCHOB2 and negative control gCHO K1 templates, but not the p425/BabV1 and p425/BabV2 plasmids, produced bands of the predicted sizes when PCR amplified using the CHO GADPH primer set (Figure 3.4 and Figure 3.5). This primer set is specific to a house keeping gene in the CHO K1 cells and used as a positive control. Genomic CHOB1 and plasmid p425/BabV1 templates produced bands of the estimated size when PCR amplified using the B1BabV primer set (Figure 3.6). The primer sets B1Conss, BCHOpolyA-1, and BCHOpolyA-2 produced bands of the predicted sizes using gCHOB1 as the template (Figure 3.7). Similarly, gCHOB2 template produced bands of the appropriate size with the B2BabV-1 primer set (Figure 3.6). When gCHOB2 template was used, bands of the predicted base pair length were produced by the B2Conss, B2BabV-2, BCHOpolyA-1, and BCHOpolyA-2 primer sets (Figure 3.8). No bands were produced by the gCHO K1 with primer sets above. The PCR products were successfully gel extracted and confirmed to contain the expected sequence via Sanger dideoxynucleotide sequencing reaction.



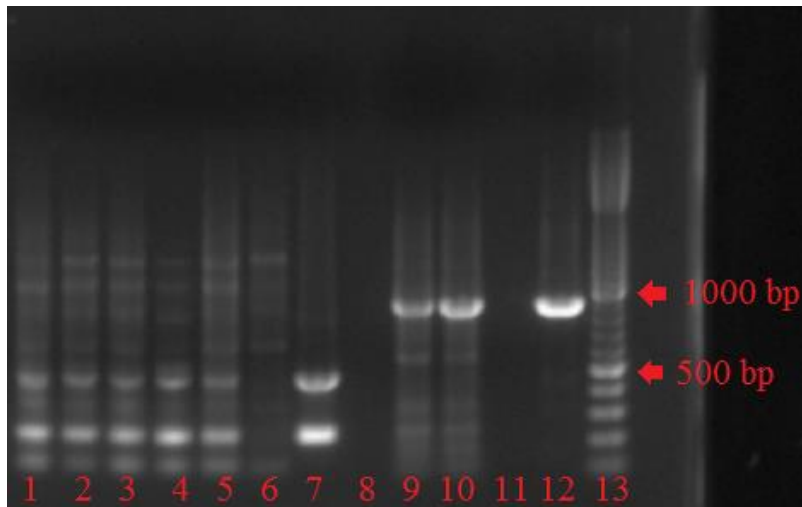
**Figure 3.4** CHOB2 Genome PCR 1

A gel electrophoresis was run on PCR products with a 100 bp ladder (5). The GADPH primer set (501 bp) was used as a positive control to confirm extraction of 1-2.) gCHOB2 DNA and 3.) gCHO K1. 4.) Plasmid p425/BabV2 was used as the negative control.



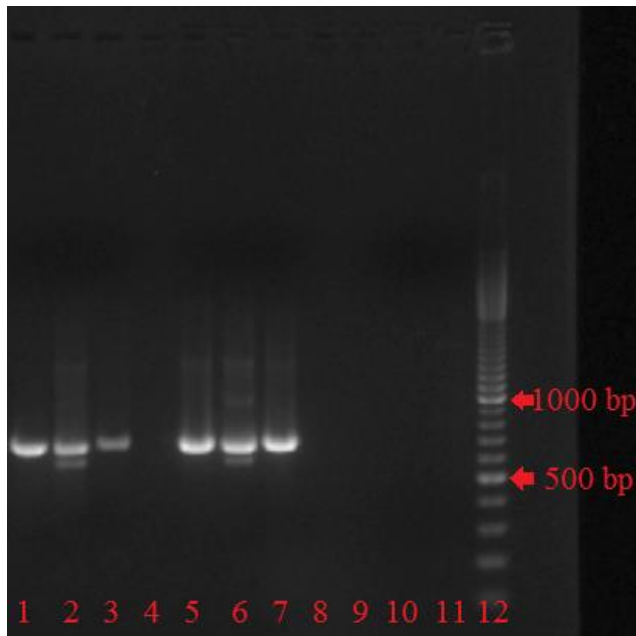
**Figure 3.5** CHOB1 Genome PCR 1

A gel electrophoresis was run on PCR products with a 100 bp ladder (7). The GADPH primer set (501 bp) was used as a positive control to confirm extraction of 2.) gCHOB1 DNA and 4.) gCHO K1. 6.) Plasmid p425/BabV1 was used as the negative control for the GADPH primer set. The products of the PCR reaction between primer set B1BabV (920 bp) of 1.) gCHOB1 and 3.) gCHO K1 DNA. The negative control for this primer set was 5.) p425/BabV1.



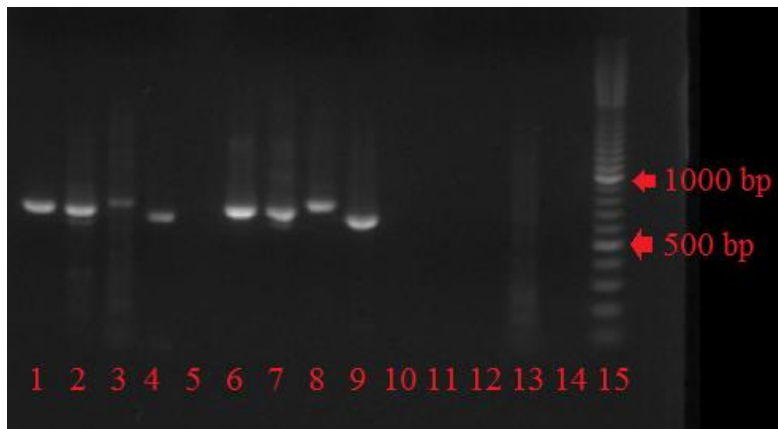
**Figure 3.6** CHOB1 and CHOB2 Genome PCR

A gel electrophoresis was run on PCR products with a 13.) 100 bp ladder. The PCR between primer set B2BabV-1 (485 bp) and templates 1-5.) gCHOB2 and the positive control 7.) p425/BabV2 indicates the presence of the *BabV2* open reading frame. The negative control 6.) gCHO K1 for this reaction did not result in a product. The PCR between primer set B1BabV (920 bp) and templates 9-10.) gCHOB1 and the positive control 12.) p425/BabV1 indicates the presence of the *BabV1* open reading frame. The negative control 11.) gCHO K1 for this reaction did not result in a product.



**Figure 3.7** CHOB1 Genome PCR 2

A gel electrophoresis was run on PCR products with a 12.) 100 bp ladder. The polyA tail of *BabV1* was confirmed to be present in gCHOB1 using primer sets 1.) BCHOpolyA-2 (688 bp) and 2.) BCHOpolyA-1 (676 bp). The conalbumin signal was confirmed to be present in gCHOB1 with the primer set 3.) B1Conss (694 bp). The positive control p425/BabV1 for these primer sets 5-7.) resulted in products of the expected sizes while the 9-11.) negative control gCHO K1 DNA did not result in products. Lanes 4.) and 8.) were intentionally left blank.

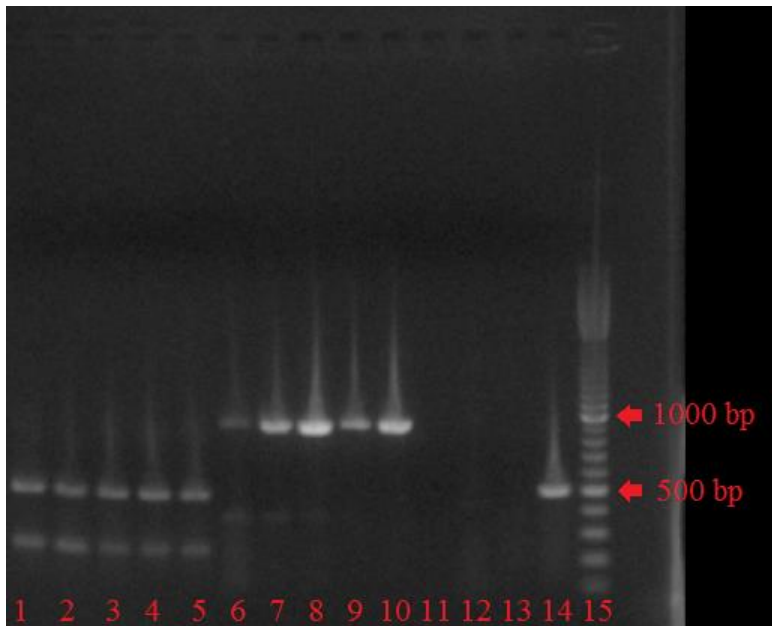


**Figure 3.8** CHOB2 Genome PCR 2

A gel electrophoresis was run on PCR products with a 15.) 100 bp ladder. The polyA tail of *BabV2* was confirmed to be present in gCHOB1 using primer sets 1.) BCHOpolyA-2 (688 bp) and 2.) BCHOpolyA-1 (676 bp). The *BabV2* open reading frame and conalbumin signal were confirmed to be present in gCHOB2 with the primer sets 3.) B2BabV-2 (730 bp) and 4.) B2Conss (636 bp). The positive control p425/BabV2 for these primer sets 6-9.) resulted in products of the expected sizes while the negative control 11-14.) gCHO K1 DNA did not result in products. Lanes 5.) and 10.) were intentionally left blank.

## RT-PCR

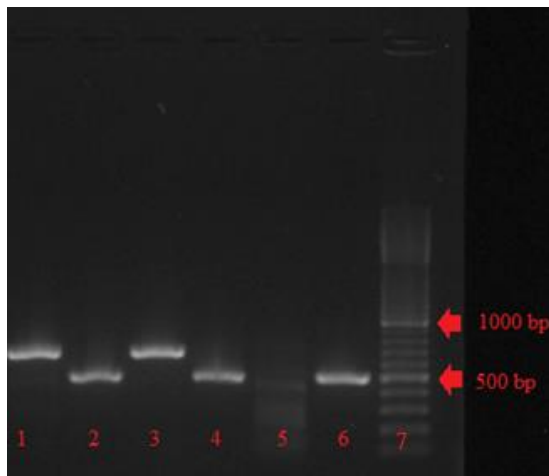
RT-PCR was used to determine if the *BabV1* and *BabV2* genes were transcribed to RNA. The RT-PCR using B1BabV and B2BabV-1 primer sets resulted in products of the expected sizes in multiple CHOB1 and CHOB2 RNA templates, respectively (Figure 3.9). The RNA template from CHOB1 and CHOB2 cells produced bands of the appropriate number of base pairs with both the CHO GADPH and BCHOpolyA-1 primer sets (Figure 3.10). Furthermore, CHOB2 RNA produced a band of the predicted size with the B2BabV-2 primer set (Figure 3.11). The CHO K1 RNA template only produced a band with the CHO GADPH primer set (Figure 3.9, Figure 3.10, and Figure 3.11). Sequencing data confirmed the PCR products contain the expected sequences.



**Figure 3.9** B1BabV and B2BabV-1 RT-PCR on CHOB1 and CHOB2 RNA

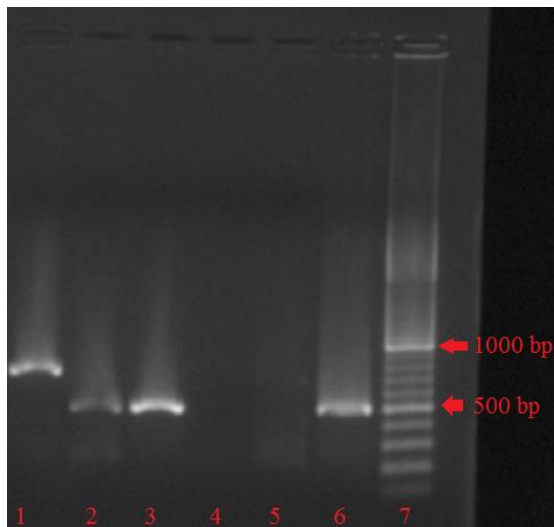
A gel electrophoresis was run on RT-PCR products with a 15.) 100 bp ladder. Wells 1-5 are the products of an RT-PCR with the primer set B2BabV-1 (485 bp) and RNA from multiple CHOB2 cultures. Wells 6-10 are the products of primer set B1BabV (920 bp) and CHOB1 RNA templates. This indicates that the open reading frames from *BabV1* and *BabV2* were transcribed to RNA in the CHOB1 and CHOB2 cells respectively. Lane 11.) was intentionally left blank. No product was produced by the RT-PCR between negative control CHO K1 RNA and primer set 12.) B2BabV-1 and 13.) B1BabV. The positive control primer set 14.) CHO GADPH (501 bp) indicates that RNA was extracted from CHO K1 cells.





**Figure 3.10** BCHOpolyA-1 RT-PCR on CHOB1 and CHOB2 RNA

A gel electrophoresis was run on RT-PCR products with a 7.) 100 bp ladder. The CHO GADPH (501 bp) primer set was used as a positive control to confirm RNA was extracted from 2.) CHOB2, 4.) CHOB1, and 6.) CHO K1. The RT-PCR with primer set BCHOpolyA-1 (676 bp) indicates RNA from the polyA tail of *BabV1* and *BabV2* is transcribed in the 3.) CHOB1 and 1.) CHOB2 cells respectively. There was no product in the RT-PCR between negative control CHO K1 RNA and 5.) BCHOpolyA-1.

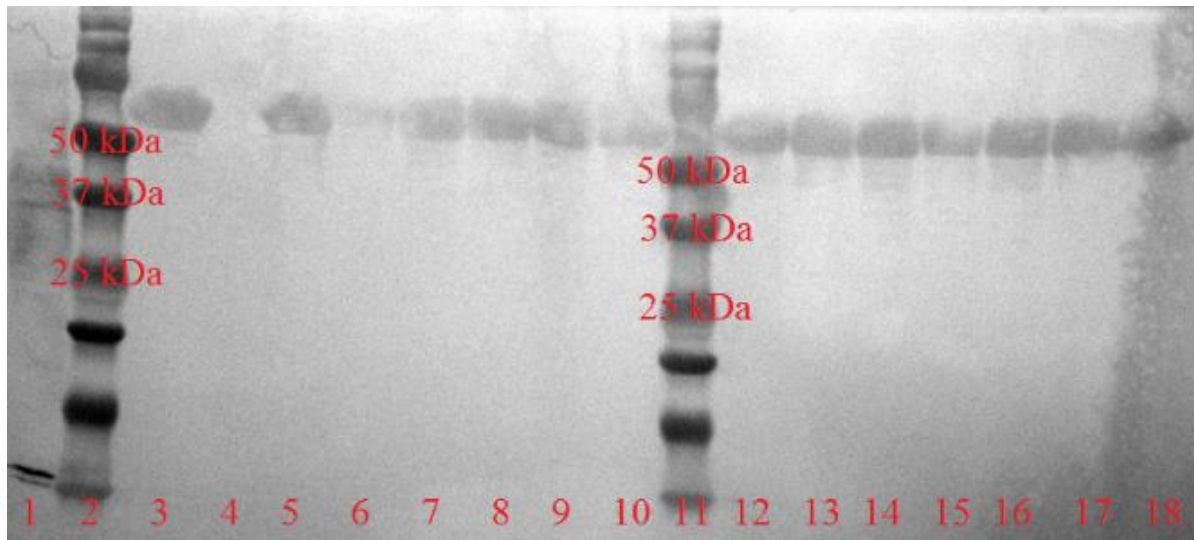


**Figure 3.11** B2BabV2 RT-PCR on CHOB2 RNA

A gel electrophoresis was run on RT-PCR products with a 7.) 100 bp ladder. The CHO GADPH (501 bp) primer set was used as a positive control to confirm RNA was extracted from 3.) CHOB2 and 6.) CHO K1. The RT-PCR between CHOB2 RNA and primer sets 1.) B2BabV-2 (730 bp) and 2.) B2BabV-1 (485 bp) indicates RNA from the open reading frame of *BabV2* was transcribed. There was no product in the RT-PCR between negative control CHO K1 RNA and primer sets 4.) B2BabV-2 and 5.) B2BabV-1.

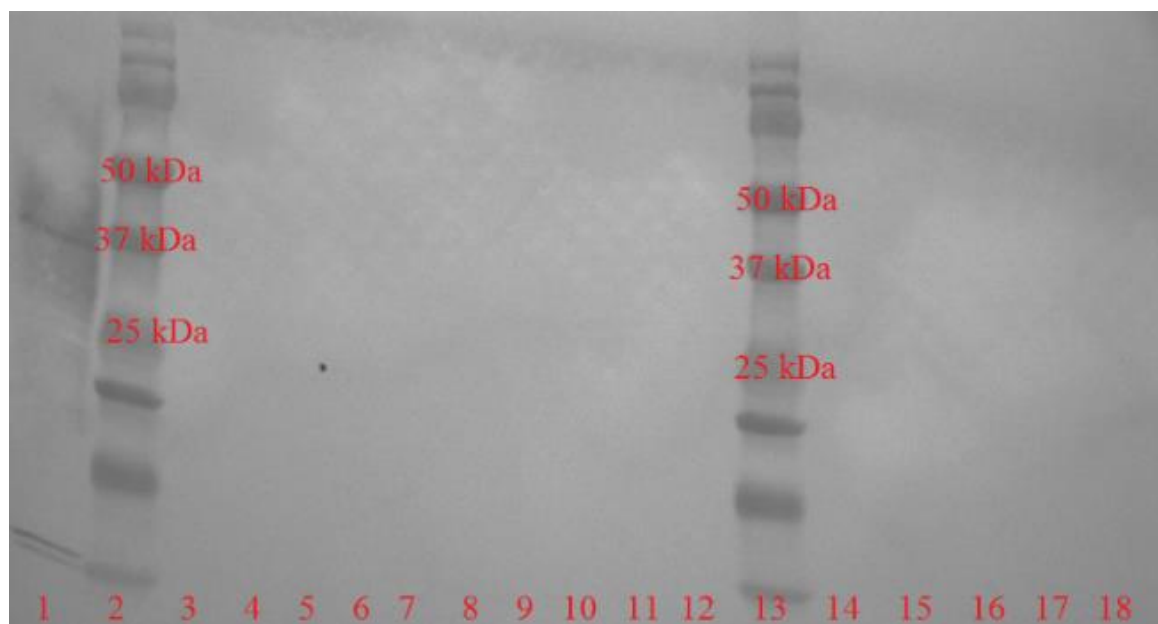
## WESTERN BLOT

Western blots were performed to determine if the rBabV1 and rBabV2 proteins were translated and recognized by the humoral immune response of *B. abortus* Strain 19 vaccinated goats. The Western blot detected bands at approximately 60 kDa in CHO K1, CHOB1, and CHOB2 serum-free media samples (Figure 3.12). Bands of varying molecular weights were detected in the *B. abortus* Strain 2308 antigen sample (Figure 3.12). Unique bands were not detected in the CHOB1 and CHOB2 samples as compared to the negative control CHO K1 media samples (Figure 3.12). The Western blot using CHOB1, CHOB2, or CHO K1 samples cell lysate samples did not detect bands (Figure 3.13). Similar to the previous Western blot, multiple bands were detected in the positive control *B. abortus* Strain 2308 antigen (Figure 3.13).



**Figure 3.12** Western Blot on Serum Free Media Samples

A Western blot using 1:7500 dilution of sera pooled from *B. abortus* Strain 19 vaccinated goats as the primary antibody. Standards are were run in lanes 2.) and 11.). The positive control 1.) killed *B. abortus* Strain 2308 antigen indicates the primary antisera is able to detect multiple *B. abortus* antigens. Serum free media cultured with non-transfected CHO K1 cells for 72 hours was used as the negative control (lanes 3. and 12.). The rBabV1 (31 kDa) and rBabV2 (32 kDa) was not detected in the 5-10.) CHOB1 and 13-18.) CHOB2 serum free media respectively. Lane 4.) was intentionally left blank.



**Figure 3.13** Western Blot on Cell Lysates

A Western blot using 1:7500 dilution of sera pooled from *B. abortus* Strain 19 vaccinated goats as the primary antibody. Standards are in lanes 2.) and 13.). The positive control 1.) killed *B. abortus* Strain 2308 antigen indicates the primary antisera detected multiple *B. abortus* antigens. Cell lysate from non-transfected CHO K1 cells was used as the negative control 3.) and 12.). The rBabV1 (31 kDa) and rBabV2 (32 kDa) was not detected in the lanes 5-10.) CHOB1 or 14-18.) CHOB2 cell lysates respectively. Lane 4.) was intentionally left blank.

## DISCUSSION

Brucellosis is a widespread zoonotic disease which causes economic burdens due to reproductive losses in animals [2, 13]. The current live-attenuated *B. abortus* vaccines do not meet the criteria for an ideal vaccine [11, 69, 70, 73]. The goal of this project was to determine if it is possible to design safe, nonliving vaccines that induce protection against *B. abortus*. The method of DNA vaccination was chosen because it is suited for inducing a cell-mediated immune response, primarily Th1 which is required for resistance to brucellosis [5, 15, 17, 35-38, 85, 86]. Two plasmids were constructed using epitopes from cellular associated antigens known to induce protection against *B. abortus* (Table 1.1). These plasmids were transfected into CHO K1 cells, and selected under puromycin pressure. The PCR on genomic DNA indicated the

p425/BabV1 and p425/BabV2 plasmids were successfully transfected into CHOB1 and CHOB2 cells, respectively (Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7, and Figure 3.8). The RT-PCR products and corresponding sequence data indicated the *BabV1* and *BabV2* genes were faithfully transcribed to RNA within the CHOB1 and CHOB2 cells, respectively (Figure 3.9, Figure 3.10, and Figure 3.11). However, Western blots using pooled *B. abortus* Strain 19 vaccinated goat sera as the primary antibody did not detect the rBabV1 or rBabV2 proteins either in the media or cell lysates (Figure 3.12 and Figure 3.13).

The pBabV1, pBabV2, and p425 plasmids were successfully digested indicating the presence of the BshTI and SalI restriction enzyme sites (Figure 3.2). The approximately 1,000 bp bands from digested pBabV1 and pBabV2 and the approximately 14,000 bp band from digested p425 were extracted and purified from the SYBR®Safe gel. These digested fragments contained a conalbumin signal sequence and BabV open reading frame. The fragments were ligated into the digested p425 which contained the 3' chicken ovalbumin polyA tail to complete the *BabV1* and *BabV2* genes. The newly constructed plasmids p425/BabV1 and p425/BabV2 were shown by gel electrophoresis to be the expected sizes of 15,277 bp and 15,250 bp, respectively (Figure 3.1). PCR reactions were performed to amplify the inserted region and polyA tail using three primer sets per plasmid (Table 2.4). The PCR products matched the expected sizes, and the successful reaction indicated primer binding sites from both the *BabV* genes and p425 were present in p425/BabV1 and p425/BabV2 (Figure 3.3). The PCR products were sequenced, confirming the expected sequence of p425/BabV1 and p425/BabV2, which includes the conalbumin signal sequence, open reading frames, and chicken ovalbumin polyA tail. The plasmids were then transfected into CHO K1 cells.

Following the introduction of 50 µg/ml puromycin to the culture media, approximately five colonies of CHOB1 and CHOB2 cells survived per well. This indicates that the concentration of puromycin used is toxic to CHO K1 cells. At five days after the transfection procedure, replicating cells were observed in the CHOB1 and CHOB2 wells but not in the untreated control wells. Taken together, this indicates that the puromycin successfully selected for transfected cells expressing the puromycin resistance gene in p425/BabV1 and p425/BabV2.

The positive control, CHO GAPDH, PCR indicated genomic DNA was successfully extracted from CHOB1, CHOB2, and CHO K1 cells (Figure 3.4 and Figure 3.5). Sequencing data from PCR products in Figure 3.5, Figure 3.6, Figure 3.7, and Figure 3.8 confirmed the conalbumin signal sequence, BabV open reading frame, and polyA tail sequence from p425/BabV1 and p425/BabV2 were present in gCHOB1 and gCHOB2 DNA, respectively. These products were not present when the negative control, gCHO K1 DNA, was used as the template, indicating the control cells were not contaminated.

To determine if the vaccine sequences were being transcribed, RT-PCR was used to detect *BabV1* and *BabV2* RNA in the CHOB1 and CHOB2 cells, respectively. The RT-PCR products from the CHO GAPDH primer set reactions with CHOB1, CHOB2, and CHO K1 indicates RNA was successfully extracted from all cultures (Figure 3.9 and Figure 3.10). The products in Figure 3.9, Figure 3.10 and Figure 3.11 indicate RNA from within the BabV open reading frame and polyA tail were transcribed in multiple CHOB1 and CHOB2 cell cultures but not the negative control CHO K1 cells. Sequencing data confirmed the RT-PCR products contained the expected sequences. Taken together, these data indicate the BabV open reading frame and polyA tail from the *BabV1* and *BabV2* genes were faithfully transcribed to RNA in the CHOB1 and CHOB2 cells, respectively.

The *BabV1* and *BabV2* genes contain the conalbumin signal sequence which directs recombinant proteins to be secreted when expressed in eukaryotic cells [177]. However, no unique bands were detected using Western blots in the CHOB1 and CHOB2 media compared to the CHO K1 media (Figure 3.12). Multiple bands were detected by the Western blots in the positive control, indicating the primary antiserum is capable of detecting *B. abortus* Strain 2308 antigens. It is possible the proteins are not being secreted despite the secretion signal. In order to test for this, cells were lysed to extract endogenous proteins for analysis.

A Western blot was performed on cell lysates to determine if the rBabV proteins were present within the CHOB1 and CHOB2 cells; however, no bands were detected in the CHOB1, CHOB2, or CHO K1 samples (Figure 3.13). The rBabV proteins should not have been degraded after lysis because a protease inhibitor cocktail was included in the lysis buffer. However, the possibility of the proteins being secreted cannot be ruled out. If this is the case, the concentration of endogenous protein may be below the detectable limits.

It is possible that the concentration of the recombinant proteins in the media and cell lysates was below the detectable limits of the Western blot. This may be overcome by concentrating the media and cell lysate samples. Alternatively, increasing the concentration of the primary antisera may detect the protein at lower concentrations. The concentration of protein in the samples may be low due to low expression levels of the *BabV1* and *BabV2* genes. The RT-PCR used in this study only detected the presence of RNA and not expression levels. A qPCR would be able to determine the expression levels of the *BabV1* and *BabV2* genes. In addition, transfecting a different cell line could increase expression of the proteins.

A limitation of Western blots is the requirement for antibodies that bind to linear epitopes, which are produced far less than antibodies which bind to conformational epitopes

[178, 179]. Therefore, using a method which recognizes conformational epitopes, such as ELISA, may detect the rBabV proteins. It has been reported that the brucella antigens Omp25 and BP26 can be detected by both ELISA and Western blots using serum from naturally infected or vaccinated animals [101, 180-182]. However, a study using anti-BP26 mAbs indicates ELISA and Western blot methods are capable of detecting different epitopes [183]. Studies indicate sera from several animal species that were naturally infected or vaccinated detects Cu/Zn SOD via Western blot [143, 180, 184, 185]. However, serum from *B. abortus* infected and Strain 19 vaccinated cows does not detect Cu/Zn SOD via ELISA [122]. This indicates several of the antigens used to construct p425/BabV1 and p425/BabV2 contain epitopes detectable by ELISA which cannot be detected via Western blot.

In this study, Strain 19 vaccinated goat sera was used as the primary antibody for the Western blots in order to determine if the BabV vaccines were recognized by the humoral response of a known effective vaccine. However, using sera from animals naturally or experimentally infected with a virulent strain may be more suitable for detecting the rBabV1 and rBabV2 proteins. It is known that the antigens recognized by serum from vaccinated and naturally infected animals differ [186]. Serum from bovines vaccinated with Strain 19 does not detect BP26 via Western blot or ELISA [60, 187]. Further, Rev 1. and M5-90 vaccinated sheep produce no or low levels of anti-BP26 antibodies [160, 188]. However, sera from naturally infected animals detect BP26 via both Western blot and ELISA [160, 188, 189]. Taken together, this suggests that the rBabV1 and rBabV2 proteins may contain epitopes recognized by antisera from animals infected with a virulent *Brucella* spp. but not vaccinated animals.

Sera from Strain 19 vaccinated goats was chosen because polyclonal antibodies binding to several epitopes would increase the chance of detection over a monoclonal antibody that only

binds to a single epitope which may not be present in the rBabV proteins. Brucella-infected sheep sera and murine monoclonal antibodies recognize the BP26 linear epitopes 93-101 and 104-111, which are not present in rBabV2 (Table 2.3) [183]. Furthermore, BP26 amino acids 230-250 have been reported to not be detected by Western blot using sera from *B. melitensis* and *B. ovis* infected sheep [182]. Future research could insert an epitope into the *BabV1* and *BabV2* genes which binds to a known monoclonal antibody. This could serve as a marker for detecting the recombinant proteins via Western blot or ELISA.

Another possible reason the Western blots did not detect the recombinant proteins is the humoral immune response may not be suitable for detecting the rBabV1 and rBabV2. Cell-mediated immunity is crucial to protection against brucellosis [5, 17, 35-38]. For this reason, T cell epitopes were selected and B cell epitopes were not intentionally included in the project design. The vaccines may not contain epitopes recognized by the humoral response. If this is the case, the cell-mediated immune response would be more likely to detect the rBabV1 and rBabV2 proteins. Numerous methods have been developed to detect T cell responses to brucella antigens. In delayed type hypersensitivity tests, brucella antigen is injected into the skin of vaccinated or naturally infected animals [40, 190]. If the antigen is recognized by T cells, inflammation occurs at the site of injection. Alternatively, splenocytes, lymph node tissue, and PBMCs from vaccinated or infected animals can be stimulated with the antigen of interest [39, 53, 67, 87, 122, 143, 144, 160, 168]. This assay has the advantage of measuring proliferation and the production of specific cytokines.



## CHAPTER IV

### SUMMARY AND CONCLUSION

#### SUMMARY

*Brucella* is a genus of Gram negative, facultative intracellular pathogens which infect 500,000 people each year globally and causes severe economic burdens in developing countries [1, 2, 12, 13]. The most effective means of controlling brucellosis is vaccination of animals [14, 15]. Live-attenuated vaccines, such as *B. abortus* Strain 19 and RB51, are used to effectively control brucellosis in cattle [5, 14]. However, these vaccines are not ideal due to residual virulence in humans, ability to cause abortion in pregnant cattle, and serological interference with diagnostic tests [14]. Multiple vaccination strategies have been researched to overcome these issues.

For a vaccine to be effective against brucella infection a cell-mediated vaccine is needed. It has been reported that the Th1 immune response must be induced for protection [5, 17, 35-38]. Cytotoxic CD8<sup>+</sup> cells are also known to play a role in protective immunity [5, 40]. Heat-killed and recombinant brucella antigens induce lower protection and a Th2 predominant immune response [84, 85]. Multiple studies have found DNA vaccination with plasmids encoding brucella antigens to be a promising immunization strategy [15, 87, 88, 94, 100-108]. In DNA vaccination, a plasmid encoding the desired antigen is injected into the host, enters the host cell and is expressed endogenously and secreted from the cell [85, 86]. This allows multiple arms of the immune system to be induced, such as Th1, Th2, and cytotoxic CD8<sup>+</sup> cells, against the expressed antigens [85, 87, 88]. DNA vaccination is considered to be a safer alternative for brucellosis vaccination [15]. Furthermore, including multiple antigens or epitopes in a vaccine has been shown to increase protection against virulent brucella challenge [15, 85-94].

## CONCLUSION

The goal of this research project was to determine if it is possible to design safe, nonliving vaccines that induce protection against *B. abortus*. Literature mining was performed to find known protective antigens. Bioinformatic technologies were used to identify the regions within these protective antigens most likely to elicit a protective immune response. The DNA sequence of these regions was used to construct two plasmids for DNA vaccination.

The p425/BabV1 and p425/BabV2 plasmids were transfected into CHO K1 cells and expressed in order to collect the recombinant protein for analysis. The genome of the transfected CHOB1 and CHOB2 cells was analyzed using PCR. Transcription of the *BabV1* and *BabV2* genes to RNA was analyzed using RT-PCR. Finally, the media and cell lysates from transfected cells were analyzed via Western blot using pooled sera from *B. abortus* Strain 19 vaccinated goats.

The PCR and RT-PCR confirmed the CHOB1 and CHOB2 cells were transfected with and transcribed RNA from the p425/BabV1 and p425/BabV2 plasmids, respectively. However, the Western blots did not detect rBabV1 or rBabV2 in the media or cell lysate. The antisera used contains antibodies specific for brucella antigens and should bind to the recombinant BabV proteins if an epitope is recognized by the humoral immune response of *B. abortus* Strain 19 vaccinated goats. No protein was detected in the 31 kDa or 32 kDa range for rBabV1 or rBabV2, respectively. It is possible that the Western blot procedure did not detect the proteins due to low protein concentration or the primary antisera not recognizing the rBabV1 or rBabV2 proteins. Future studies will need to concentrate the protein, use different antisera, or utilize ELISA. Another possible route is to use assays which detect cell-mediated immune responses such as delayed type hypersensitivity or splenocyte assays.

## APPENDIX A PROTOCOLS

### Anza Restriction Digest (Thermofisher Scientific) Citation [191]

1. Prepare a reaction mix by adding reagents in the order indicated in Table 1.
2. Incubate at 37°C for 15 minutes.

**Table 1**

Reagent	1 enzyme reaction	2 enzyme reaction	3 enzyme reaction
Nuclease-free water	As required to make up final reaction volume		
Anza 10x Buffer or Anza 10X Red Buffer	2 µL	2 µL	3 µL
DNA	0.2–1 µg	0.2–1 µg	0.2–1 µg
Anza restriction enzyme 1	1 µL	1 µL	1 µL
Anza restriction enzyme 2	—	1 µL	1 µL
Anza restriction enzyme 3	—	—	1 µL
Final reaction volume	20 µL	20 µL	30 µL

Volumes can be scaled up linearly to 5X.

**Zymoclean™ Gel DNA Recovery Kit (Zymo Research)** Citation [192]

1. Excise the DNA fragment<sup>1</sup> from the agarose gel using a razor blade, scalpel or other device and transfer it into a 1.5 ml microcentrifuge tube.
2. Add 3 volumes of **ADB** to each volume of agarose excised from the gel (*e.g.* for 100 µl (mg) of agarose gel slice add 300 µl of **ADB**). Incubate at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved<sup>2</sup>.
3. For DNA fragments > 8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (*e.g.*, 100 µl agarose, 300 µl **ADB**, and 100 µl water).
4. Transfer the melted agarose solution to a **Zymo-Spin™ Column** in a **Collection Tube**.
5. Centrifuge for 30-60 seconds. Discard the flow-through<sup>3</sup>.
6. Add 200 µl of **DNA Wash Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add ≥ 6 µl DNA Elution Buffer<sup>4</sup> or water<sup>5</sup> directly to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA. Ultra-pure DNA is now ready for use.

**Quick Ligase™ (New England Biolabs) Citation [193]**

1. Set up the following reaction in a microcentrifuge tube on ice.

*(T7 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.)*

Use NEBiolcalculator to calculate molar ratios.

COMPONENT	20 µl REACTION
Quick Ligase Reaction Buffer (2X)*	10 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free Water	to 20 µl
Quick Ligase	1 µl

*\*The Quick Ligase Reaction Buffer should be thawed and resuspended at room temperature.*

2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. Incubate at room temperature (25°C) for 5 minutes.
4. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells. Alternatively, Store at -20°C.
5. Do not heat inactivate – heat inactivation dramatically reduces transformation efficiency.

**AccuPrime™ DNA polymerase enzyme and buffer (Thermofisher Scientific) Citation [194]**

Add the following components to a DNase/RNase-free, thin-walled PCR tube. For multiple reactions, prepare a master mix of common components to minimize reagent loss and enable accurate pipetting.

Component	Amount for one 50-μL reaction	
	Plasmids/cDNA/ DNA	Genomic DNA
10X AccuPrime™ PCR Buffer I	5 μL	—
10X AccuPrime™ PCR Buffer II	—	5 μL
Sense primer (10 μM)	1 μL	1 μL
Anti-sense primer (10 μM)	1 μL	1 μL
Template DNA	0.1 pg to 20 ng	10 pg to 500 ng
AccuPrime™ <i>Taq</i> DNA Polymerase, High Fidelity	0.2 μL	0.2 μL
Autoclaved, distilled water	to 50 μL	to 50 μL

2. Cap the tube, tap gently to mix, then centrifuge briefly to collect the contents.

3. Place the tube in the thermal cycler, then run the following program:

Step	Temperature	Time
Initial denaturation 25–35 cycles of:	94°C	15 seconds to 2 minutes
Denature	94°C	15–30 seconds
Anneal	52–64°C	15–30 seconds
Extend	68°C	1 minute per kb

4. Maintain the reactions at 4°C after cycling. Samples can be stored at –20°C until use.
5. Analyze the amplification products by agarose gel electrophoresis.

**Invitrogen MAX Efficiency® Stbl2™ Competent cells (ThermoFisher Scientific) Citation [195]**

1. Thaw competent cells on wet ice. Place the required number of 17 × 100 mm Falcon® 2059 tubes or similarly shaped polypropylene tubes (see the following note) on ice.
2. Gently mix the cells, then aliquot 100 µL of competent cells into chilled tubes.
3. Refreeze any unused cells in the dry ice/ethanol bath for 5 minutes before returning them to the –85°C to –68°C freezer. Do not use liquid nitrogen.
4. To determine transformation efficiency, add 5 µL (50 pg) control DNA to one tube containing 100 µL competent cells. Move the pipette through the cells while dispensing. Gently tap the tube to mix.
5. For DNA from ligation reactions, dilute the reactions 5-fold in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Add 1 µL of the dilution to the cells (1–10 ng DNA), moving the pipette through the cells while dispensing the dilution. Gently tap the tubes to mix.
6. Incubate the cells on ice for 30 minutes.
7. Heat-shock the cells 25 seconds in a 42°C water bath; do not shake the cells.
8. Place on the cells ice for 2 minutes.
9. Add 0.9 mL of room-temperature S.O.C. Medium (Cat. no. 15544-034).
10. Shake the tubes containing ligation reaction at 225 rpm (30°C) for 90 minutes.



### **ZymoPURE™ Plasmid Midiprep (Zymo Research) Citation [196]**

1. Add 8 ml of ZymoPURE™ P1 (Red) to the bacterial cell pellet and resuspend completely by vortexing or pipetting.
2. Add 8 ml of ZymoPURE™ P2 (Green) and immediately mix by gently inverting the tube 6 times. Do not vortex! Let sit at room temperature for 2-3 minutes. Cells are completely lysed when the solution appears clear, purple, and viscous.
3. Add 8 ml of ZymoPURE™ P3 (Yellow) and mix gently but thoroughly by inversion. Do not vortex! The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form.
4. Ensure the plug is attached to the Luer Lock at the bottom of the ZymoPURE™ Syringe Filter. Place the syringe filter upright in a tube rack and load the lysate into the ZymoPURE™ Syringe Filter and wait 5 - 8 minutes for the precipitate to float to the top.
5. Remove the Luer Lock plug from the bottom of the syringe and place it into a clean 50 ml conical tube. Place the plunger in the syringe and push the solution through the ZymoPURE™ Syringe Filter to clear the debris. Save the cleared lysate!
6. Add 8 ml ZymoPURE™ Binding Buffer to the cleared lysate from step 6 and mix thoroughly by inverting the capped tube 8 times.

<b>Vacuum Protocol</b>	<b>Centrifugation protocol</b>
The vacuum pump should be a single or double-staged unit capable of producing up to 400 mm Hg pressure.	A swinging bucket rotor is required for centrifugation.
8. Ensure the connections of the Zymo-Spin™ III-P Column	8. Remove the 50 ml Reservoir from the top of the Zymo-Spin™ III-P Column Assembly. Ensure the connection between the 15 ml Conical Reservoir and Zymo-Spin™ III-P column is

Assembly are finger-tight and place onto a vacuum manifold.

9. Add the entire mixture from step 7 into the Zymo-Spin™ III-P Column Assembly, and then turn on the vacuum until all of the liquid has passed completely through the column.

10. Unscrew the purple Luer Lock cap from the top of the Zymo-Spin™ III-P Column and discard the Reservoirs.

finger-tight and place the assembly into a 50 ml conical tube.

9. Add 10 ml of the mixture from step 7 into the **15 ml Conical Reservoir/Zymo-Spin™ III-P Column** assembly, and centrifuge at 500 x g for 2 minutes.

10. Empty the 50 ml conical tube and repeat step 9 until the entire sample has passed through the column.

**Note:** Steps 11-12 can also be completed using a microcentrifuge instead of the vacuum manifold (see full instruction manual).

11. With the vacuum off, add 800 µl of ZymoPURE™ Wash 1 to the Zymo-Spin™ III-P Column. Turn on the vacuum until all of the liquid has passed completely through the column.

12. With the vacuum off, add 800 µl of ZymoPURE™ Wash 2 to the Zymo-Spin™ III-P Column. Turn on the vacuum until all of the liquid has passed completely through the column. Repeat this wash step.

13. Place the Zymo-Spin™ III-P Column in a Collection Tube and transfer to a microcentrifuge. Centrifuge at  $\geq 10,000 \times g$  for 1 minute in order to remove any residual wash buffer. Transfer the Zymo-Spin™ III-P Column into a clean 1.5 ml tube and add 200 µl of ZymoPURE™ Elution Buffer directly to the column matrix. Incubate at room temperature for 2 minutes, and then centrifuge at  $\geq 10,000 \times g$  for 1 minute in a microcentrifuge.

### **Zyppy™ Plasmid Miniprep (Zymo Research) Citation [197]**

1. Add 600 µl of bacterial culture grown in LB medium to a 1.5 ml microcentrifuge tube.
2. Add 100 µl of **7X Lysis Buffer (Blue)** and mix by inverting the tube 4-6 times. Proceed to step 3 within 2 minutes.
3. Add 350 µl of cold **Neutralization Buffer (Yellow)** and mix thoroughly. **Invert the sample an additional 2-3 times** to ensure complete neutralization.
4. Centrifuge at 11,000 – 16,000 x g for 2-4 minutes.
5. Transfer the supernatant (~900 µl) into the provided **Zymo-Spin™ IIN** column. Avoid disturbing the cell debris pellet.
6. Place the column into a **Collection Tube** and centrifuge for 15 seconds.
7. Discard the flow-through and place the column back into the same **Collection Tube**.
8. Add 200 µl of **Endo-Wash Buffer** to the column. Centrifuge for 30 seconds.
9. Add 400 µl of **Zyppy™ Wash Buffer** to the column. Centrifuge for 1 minute.
10. Transfer the column into a clean 1.5 ml microcentrifuge tube then add 30 µl of **Zyppy™ Elution Buffer** directly to the column matrix and let stand for one minute at room temperature.
11. Centrifuge for 30 seconds to elute the plasmid DNA.

**TrypLE™ Express (Thermo Fisher Scientific) Citation [198]**

1. Pre-warmed TrypLE™ and complete growth medium to 37°C before use. Minimize dwell time.

Note: TrypLE™ may be used at ambient room temperature for many types of cells.

2. Aspirated spent medium and discarded.

3. Washed cell monolayer with 5 mL of Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium. Aspirated and discarded.

4. Added 0.5 ml or 1 ml of TrypLE™ to well or flask, respectively. Ensure complete coverage of cell monolayer with TrypLE™.

5. Incubated at 37°C until cells have detached. Observe cell monolayer using an inverted microscope to ensure complete cell detachment from the surface of the flask. Gently tap flask to dislodge cells if necessary.

6. Added 5–10 mL of pre-warmed complete medium to flask. Tilt flask in all directions to thoroughly rinse flask. Transfer cell suspension to a 15-mL conical tube.

7. Centrifuged at  $100 \times g$  for 5–10 minutes.

8. Discarded supernatant and resuspended cell pellet with 2–5 mL of pre-warmed complete medium.

9. Seed, incubate and subculture according to normal protocols depending on your cell type.

**Lipofectamine™ 3000 (Thermo Fisher Scientific) Citation [199]**

Concentration of plasmid DNA:

BabV1/425 = 1928 ng/μl

BabV2/425 = 2794 ng/μl

Transfection procedure:

1. 3 six well plates were obtained. Labeled CNTL, B1 or B2.
2. CHO K1 cells were split using TrypLE™ and aliquoted into the wells of the plate
3. Cells grown to 70-90% confluency
4. Cells were washed and fed with SF media + 3% FBS + Glutamax before transfection

Per plate:

5. 375 μl of SF media + Glutamax mixed with 11.25 or 22.5 μl of lipofectamine 3000.  
Vortexed briefly
6. 750 μl of SF media + Glutamax mixed with 15 μg DNA (7.5 μl BabV1/425 or 5.4 μl BabV2/425) and 30 μl P3000 reagent. Vortexed
7. High and low concentrations were made by mixing 1:1 ratio of the lipofectamine and DNA mixtures.
8. Mixture incubated for 10-15 minutes
9. Cells were washed then fed with 250 μl of the final mixture
10. Incubated for 4 days then washed, fed and analyzed for transfection success
11. \*\*no DNA was added to the mixture for CNTL cells

### **M-Per Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) [200]**

Prior to the procedure, 250  $\mu$ l of M250 Protease Inhibitor Cocktail, Mammalian (Amresco, Solon, Ohio) was added to 25 ml M-Per Reagent.

1. Thaw and pellet the cells by centrifugation at  $2500 \times g$  for 10 minutes. Discard the supernatant.
2. Wash the cells once by resuspending the cell pellet in wash buffer (e.g., PBS). Pellet cells by centrifugation at  $2500 \times g$  for 10 minutes.
3. Add 300 $\mu$ l M-PER Reagent to the cell pellet. Pipette the mixture up and down to resuspend pellet.
4. Shake mixture gently for 10 minutes. Remove cell debris by centrifugation at  $\sim 14,000 \times g$  for 15 minutes.
5. Transfer the supernatant to a new tube for analysis.

**DNEasy® Blood and Tissue Kit (QIAGEN) Citation [201]**

1. Cultured cells: Centrifuge the appropriate number of cells (maximum  $5 \times 10^6$ ) for 5 min at 300 x g. Resuspend the pellet in 200  $\mu$ l PBS. Add 20  $\mu$ l proteinase K.

Continue with step 2.

When using a frozen cell pellet, allow cells to thaw before adding PBS until the pellet can be dislodged by gently flicking the tube. Ensure that an appropriate number of cells is used in the procedure. Add 4  $\mu$ l RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature before continuing with step 2.

2. Add 200  $\mu$ l Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 10 min. It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

3. Add 200  $\mu$ l ethanol (96–100%) to the sample, and mix thoroughly by vortexing. It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

4. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500  $\mu$ l Buffer AW1, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard flow-through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500  $\mu$ l Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube. It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This

centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute. Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield.



**RNEasy® Plus Micro Kit (QIAGEN) Citation [202]**

1. Cell samples were thawed and centrifuged at 300 x g for 5 minutes. Supernatant was aspirated off and discarded.

2. Cells disrupted cells by adding Buffer RLT Plus.

For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add 350 µl Buffer RLT Plus. Vortex or pipet to mix, and proceed to step 3.

3. Homogenized the lysate by vortexing

4. Lysate was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). Centrifuge for 30 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm). Discarded the column, and saved the flow through.

5. Added 350 µl of 70% ethanol to the flow-through from step 4, and mixed well by pipetting.

Do not centrifuge. Proceed immediately to step 6.

6. Transferred the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Closed the lid gently, and centrifuged for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm). Discard the flow-through.\*

Reuse the collection tube in step 7.

7. Added 700 µl Buffer RW1 to the RNeasy MinElute spin column. Closed the lid gently, and centrifuged for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash the spin column membrane. Discard the flow-through.\*

Reuse the collection tube in step 8.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

8. Added 500  $\mu$ l Buffer RPE to the RNeasy MinElute spin column. Closed the lid gently, and centrifuged for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash the spin column membrane. Discard the flow-through.

Reuse the collection tube in step 9.

9. Added 500  $\mu$ l of 80% ethanol to the RNeasy MinElute spin column. Closed the lid gently, and centrifuged for 2 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.

Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

10. Placed the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discarded the collection tube with the flow-through.

11. Placed the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14  $\mu$ l RNase-free water directly to the center of the spin column membrane. Closed the lid gently, and centrifuged for 1 min at full speed to elute the RNA.

### OneStep RT-PCR (QIAGEN) Citation [203]

1. Thaw template RNA, primer solutions, dNTP Mix, 5x QIAGEN OneStep RT-PCR Buffer, and RNase-free water, and place them on ice.
2. Prepare a master mix according to the table below.

	Volume
RNase free water	39 $\mu$ l
5x QIAGEN OneStep RT-PCR Buffer	10 $\mu$ l
dNTP Mix	2 $\mu$ l
F Primer	3 $\mu$ l
R Primer	3 $\mu$ l
QIAGEN OneStep RT-PCR Enzyme Mix	2 $\mu$ l
Template RNA	1 $\mu$ l
<b>Total</b>	50 $\mu$ l

3. Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes. Mix gently, for example, by pipetting the master mix up and down a few times.
4. Add template RNA ( $\leq 2$   $\mu$ g/reaction) to the individual PCR tubes.
5. Program the thermal cycler according to the program outlined in the table below.

<b>Reverse Transcription</b>	30 minutes	50°C
<b>Initial PCR activation</b>	15 minutes	95°C
<b>3 Step cycling</b>		
Denaturation	30-60 seconds	94°C
Annealing	30-60 seconds	50-68°C
Extension	1 minute	72°C
<b>Number of cycles</b>	25-40 cycles	
<b>Final extension</b>	10 minutes	72°C

6. Start the RT-PCR program while PCR tubes are still on ice. Wait until the thermal cycler has reached 50°C. Then place the PCR tubes in the thermal cycler.

## **APPENDIX B**

### **PILOT STUDY USING BRUCELLA ABORTUS STRAIN 19 AS A MODEL FOR VIRULENT CHALLENGE IN GOATS**

*Brucella* is a genus of Gram negative, facultative intracellular pathogens named for their natural hosts with *B. melitensis* for goats and *B. abortus* for cattle [1, 5]. Infected cattle abort during the last trimester of pregnancy and secrete *Brucella* in milk [2, 68]. Human exposure occurs through unpasteurized milk, blood, lymph or tissues from infected animals and may result in undulant fever, arthritis, endocarditis, and spondylitis [2-4, 6, 11]. Globally, there are approximately 500,000 cases of human brucellosis reported each year and high economic burdens due to animal brucellosis in developing countries [12, 13]. In the United States, there are limited means of controlling brucellosis in wildlife reservoirs which are capable of spreading infection to domestic livestock [17, 204].

Vaccination has proven to be effective in controlling brucellosis; however, further research is necessary to develop an ‘ideal’ vaccine [11, 14, 15, 69, 70]. While the mouse is the standard animal model for brucellosis vaccine development, results are not always applicable to the target species, such as cattle [17]. The caprine model has the advantage of using both a ruminant and natural host for *Brucella* spp. [68]. Goats infected with *B. abortus* develop similar clinical signs and serological response as cattle [205]. Goats are also less costly to maintain and have shorter gestation lengths than large ruminants [68]. Vaccine development is further complicated because the challenge strain *B. abortus* 2308 is a select agent, which is subject to strict regulations [206]. A possible solution to this obstacle is using *Brucella* spp. that are exempt from these regulations as the challenge, such as Strain 19 [207].

The first widely used vaccine against bovine brucellosis in the U.S. was the live-attenuated *B. abortus* Strain 19, which provides protection for the entire productive life span of

the animal [14, 69]. In domestic cattle, vaccination with Strain 19 combined with test and slaughter policies has been effective in controlling brucellosis in several countries [208, 209]. While less protective, it can be used in wildlife control plans, as well [210-213]. Despite these benefits, Strain 19 has many negative qualities. Vaccination with Strain 19 interferes with serological tests, which led to it being replaced by Strain RB51 in the United States [69, 73].

Strain 19 also maintains a low level of virulence, may cause abortion in pregnant cattle, and remains pathogenic to humans [69]. Vaccination with Strain 19 is also known to cause orchitis and can be isolated from testicles of bulls [214, 215]. Though it is rare, some cattle remain chronically infected and excrete Strain 19 in milk [69, 209, 216]. The rate of chronic infection and abortion is higher in bison, which limits its use in wildlife control plans [212].

It is known that goats abort and bacteria can be isolated from milk and fetal tissues when vaccinated with *B. melitensis* Rev. 1, another live-attenuated strain, during late pregnancy [217-220]. Furthermore, abortion rate and shedding increases in a dose dependent manner with higher doses of Rev. 1 [219]. Due to its residual virulence and ability to infect ruminants other than cattle, we hypothesize that a sufficient dose of Strain 19 may mimic virulent challenge in both non-pregnant and pregnant goats.

## **MATERIALS AND METHODS**

### **STRAIN 19 AND BACTERIAL CULTURE**

*Brucella abortus* Strain 19 was obtained from laboratory stocks originally from the USDA and kept at -80°C. Bacteria were grown on Schaedler agar (Remel, San Diego, CA) with Brucella Selective Supplement (Oxoid Inc, Nepean, ON) and 5% Horse serum (Sigma Life Science, St. Louis, MO) and incubated at 37°C with 5% CO<sub>2</sub> and humidity. Inoculation doses of Strain 19 were grown on the plates then collected by washing with PBS and pipetting off the liquid into vials. The vials were confirmed to contain 1X10<sup>11</sup> colony forming units (CFU)/ml by

serial dilution. Vials were snap frozen and stored at -80°C until time of infection. Before infection, vials were thawed and diluted in PBS to the appropriate concentrations and CFU/ml confirmed by serial dilutions.

## **GOATS**

All goats were bled, Card tested, and dewormed prior to infection. For the transient colonization study, eight breeding age female Boer goats were housed at the Agriculture Center Isolation Facility (ACIF, Baton Rouge, LA) and acclimated for two weeks before infection. Goats were infected with either  $1 \times 10^9$  or  $1 \times 10^{10}$  CFU by placing 50  $\mu$ l inoculum in each conjunctival sac. At two and four weeks post infection, two goats from each group were sacrificed by captive bolt and exsanguination. Blood, parotid lymph node, prescapular lymph node, internal iliac lymph node, supramammary lymph node, liver, and spleen samples were taken at the time of sacrifice. Tissue samples were stored at -20°C until testing.

All goats were bled, Card tested and dewormed prior to infection and exposure to bucks. Does were exposed to two bucks for 60 days. Pregnancy status and gestation length were confirmed by ultrasound. Thirteen pregnant does were housed at ACIF and acclimated for two weeks prior to infection at approximately 100 days gestation. The treatment groups (4 goats each) were infected with either  $1 \times 10^9$  or  $1 \times 10^{10}$  CFU via 50  $\mu$ l inoculum in each conjunctival sac (CJ). The positive control group (5 goats) was infected with  $1 \times 10^7$  CFU via intravenous (IV) injection. Goats were monitored twice a day, and parturition status was recorded as live or dead. Kids were euthanized by CO<sub>2</sub> asphyxiation and exsanguination immediately after parturition. Fetal lung, spleen and abomasal fluid samples were collected and stored at -20°C until testing. Milk samples were taken 2-4 weeks post-parturition and stored at -20°C until testing. Maternal blood samples were taken at 2-4 weeks and 4-6 weeks post parturition.

## **SERA, MILK AND TISSUE SAMPLES**

Blood samples were allowed to clot at room temperature then centrifuged at 3400g, and sera was pipetted off and stored at -20°C until testing. Card tests were performed on sera samples to determine serological status. Lymph node, spleen and liver samples were thawed; and ground with a Bullet Blender 50 (Next Advance, Averill Park, NY); and 100 µl aliquots were plated. Aliquots of abomasal fluid and milk were plated (100 µl). Fetal lung samples were tested for bacteria by blotting. Briefly, lung samples were dipped in 70% ethanol then blotted dry on sterile gauze. Lung tissue was cut with sterile scissors and the exposed tissue pressed against the Schaedler agar. Final CFU/ml and CFU/gram of tissue were determined after three weeks of incubation.

## **BRUCELLA IDENTIFICATION**

Suspected *B. abortus* Strain 19 colonies were confirmed by Gram stain, colony morphology, oxidase, catalase, urease, H<sub>2</sub>S and TSI biochemical tests.

## **STATISTICS**

Statistical analysis was not used because this was a pilot study, and numbers were kept small.

## **RESULTS**

### **TRANSIENT COLONIZATION**

All goats were Card negative pre-infection. Post-infection, one goat challenged with 1X10<sup>9</sup> CFU was Card negative while all others tested positive (Table 3). At 14 days post infection, Strain 19 was isolated from all lymph node, spleen, and liver samples of the 1X10<sup>10</sup> group, while the 1X10<sup>9</sup> group only displayed bacteria in the parotid lymph node (Table 1 and Table 2). At 28 days post infection, one goat in the 1X10<sup>9</sup> group was colonized in all tissue samples taken, while the other goat in the group only had Strain 19 recovered from the parotid

lymph node. In the  $1 \times 10^{10}$  group, the prescapular lymph node, supramammary lymph node, and spleen were colonized in only one goat (Table 2).

$1 \times 10^9$	Par	PS	SM	II	Spl	LV
14 days	250 (2.40)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
14 days	121.28 (2.08)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
28 days	287.88 (2.46)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
28 days	7320.2 (3.86)	225.42 (2.35)	95.32 (1.98)	109.2 (2.04)	108.69 (2.04)	28.27 (1.45)

**Table 1.**

CFU/g (Log/g of tissue) of *B. abortus* Strain 19 recovered from tissue of goats infected with  $1 \times 10^9$  at 14 and 28 days postinfection. Parotid lymph node (Par), Prescapular lymph node (PS), supramammary lymph node (SM), internal iliac lymph node (II), spleen (Spl), and liver (LV) samples.

$1 \times 10^{10}$	Par	PS	SM	II	Spl	LV
14 days	9223.3 (3.96)	84.82 (1.93)	143.94 (2.16)	120.25 (2.08)	8520.18 (3.93)	0 (0)
14 days	2900.76 (3.46)	201.7 (2.3)	246.75 (2.39)	226.19 (2.35)	1187.5 (3.07)	23.17 (1.36)
28 days	604.05 (2.78)	0 (0)	0 (0)	0 (0)	27.14 (1.43)	0 (0)
28 days	7587.86 (3.88)	791.67 (2.90)	284.43 (2.45)	0 (0)	0 (0)	0 (0)

**Table 2.**

CFU/g (Log/g of tissue) of *B. abortus* Strain 19 recovered from tissue goats infected with  $1 \times 10^{10}$  at 14 and 28 days postinfection. Parotid lymph node (Par), Prescapular lymph node (PS), supramammary lymph node (SM), internal iliac lymph node (II), spleen (Spl), and liver (LV) samples.

	14 days	28 days
$1 \times 10^9$	50%	100%
$1 \times 10^{10}$	100%	100%

**Table 3.**

Percent of Card positive nonpregnant goats at 14 and 28 days postinfection with  $1 \times 10^9$  or  $1 \times 10^{10}$  CFU of *B. abortus* Strain 19.

## PREGNANT CHALLENGE

All does were Rose Bengal card test negative prior to infection. Table 4 shows the serological status of the does at 2-4 and 4-6 weeks post parturition. Sonograms were used to estimate gestation length within 21 days. Average gestation lengths (Table 5) are within 21 days



of the average 150 days of normal caprine gestation. All kids were born alive; and no Strain 19 was recovered from any of the milk, fetal lung, fetal spleen, or abomasal fluid samples (data not shown).

	CJ 10 <sup>9</sup>	CJ 10 <sup>10</sup>	IV 10 <sup>7</sup>
2-4 weeks	50%	75%	50%
4-6 weeks	50%	66%	50%

**Table 4.**

Percent Card positive at 2-4 and 4-6 weeks post parturition. Goats were infected at approximately 100 days gestation with *B. abortus* Strain 19 either conjunctively (CJ) or intravenously (IV).

	Average Gestation Length (Days)
1X10 <sup>9</sup> CJ	129.25
1X10 <sup>10</sup> CJ	133.25
1X10 <sup>7</sup> IV	129.25

**Table 5.**

Estimated gestation length based on sonograms. All groups are within the normal gestation length of 150 days for caprine pregnancy.

## DISCUSSION

The transient colonization study performed suggests that Strain 19 colonization is consistent with the profile of an attenuated mutant in the caprine model [68]. The liver, spleen, and all lymph nodes were colonized by 14 days post infection in the 1X10<sup>10</sup> CFU group (Table 2). In contrast to virulent *Brucella* strains [68], Strain 19 was clearing from the animal by 28 days post-infection (Table 2). The lower dose, 1X10<sup>9</sup> CFU, disseminated slowly and was only detectable beyond the parotid lymph node in one goat sacrificed at 28 days post infection (Table 1). All animals except one were Card positive at time of sacrifice (Table 3). The Card negative animal was from the 1X10<sup>9</sup> CFU group sacrificed at 14 days post inoculation, and Strain 19 had not disseminated to tissues beyond the parotid lymph node.

The virulent challenge *B. abortus* caprine model produces consistent results in pregnant goats; 30-50% abort and 50-70% dam kid pair colonization [68]. Further, goats infected with *B. abortus* 2308 during the third trimester of pregnancy shed bacteria in milk [221]. The Card test indicates that 50-75% of the pregnant goats were transiently colonized by Strain 19 (Table 4). However, the conjunctival and intravenous challenge with attenuated Strain 19 resulted in no abortions; and bacteria was not recovered from milk, fetal tissue, or abomasal fluid samples (Table 4 and Table 5).

The inability of *B. abortus* Strain 19 to induce abortion might be due to infection occurring in a non-native host. It has been shown that *B. abortus* induces less lesions in pregnant goats than pregnant cows [222]. Furthermore, virulent *B. melitensis* challenge results in both higher abortion (70-100%) and dam kid pair colonization (90-100%) rates than *B. abortus* in the caprine model [68]. Based on previous research, the live-attenuated vaccine *B. melitensis* Rev. 1 persists in goats longer than *B. abortus* Strain 19 [223]. Three weeks after vaccination, Rev. 1 can be isolated from the spleen, liver and systemic lymph nodes and begins to clear between weeks three and five. The bacteria persist only in the prefemoral and prescapular lymph nodes after eight weeks [223]. Despite not being directly comparable, our data suggests that *B. abortus* Strain 19 and *B. melitensis* Rev. 1 are in a similar stage of clearance from the goat at four and eight weeks post infection, respectively [223] (Table 4 and Table 5).

The intravenous inoculation route immediately induces bacteremia in the animal, suggesting that Strain 19 was not able to cross the caprine placenta or crossed the placenta but failed to colonize the fetus. Studies indicate that complications due to Strain 19 vaccination in cows are rare. The vaccine strain induces abortion and is shed in the milk of only 1-5% and

1.9% of vaccinated cows, respectively [224-227]. Our results suggest that Strain 19 is similarly attenuated in the caprine model.

## CONCLUSION

Animal brucellosis causes high economic burdens and infects over half a million people a year worldwide [12, 13]. Development of improved vaccines is limited by regulations on the virulent strains of *Brucella* spp [206]. The purpose of this study was to determine if the live-attenuated vaccine Strain 19 could mimic a virulent *B. abortus* challenge in the caprine model. Transient colonization was established in non-pregnant goats, with the higher inoculation dose disseminating more rapidly and bacteria clearing from the animals by 28 days post challenge (Table 1 and 2). Pregnant goats challenged with Strain 19 did not abort; and no bacteria were recovered from milk, fetal lung, spleen, or abomasal fluid. This suggests that *B. abortus* Strain 19 is not able to act as a virulent challenge in pregnant goats.

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