CHARACTERIZATION OF THE CELLULAR IMMUNE RESPONSE TO TONB-DEPENDENT PROTEINS OF *Histophilus somni* IN MURINE AND CATTLE MODELS.

A Thesis

Submitted to the Faculty of Graduate Studies and Research

In Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the Department of Veterinary Microbiology

University of Saskatchewan

Saskatoon

By

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Fall, 2016

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ABSTRACT

Histophilus somni is an economically important member of the bovine respiratory disease (BRD) complex. An *H. somni* infection has a number of clinical manifestations including septicemia, myocarditis, pneumonia, bronchopneumonia, arthritis and infectious thromboembolic meningoencephalitis. The incidence of systemic disease has significantly increased over the past decade while respiratory disease incidence has remained constant. Current vaccines based on decades old strains are less effective against the systemic form of disease.

An understanding of the immune responses generated as well as the antigens expressed during infection is essential in producing effective vaccines. TonB-dependent proteins have been shown to be protective in other members of the *Pastuerellaceae* family. Therefore, I hypothesized that vaccination with *H. somni* TonB-dependent proteins would be protective against experimental infection and a good cellular immune response would be induced following immunization.

The genes coding for the TonB-dependent receptor 1372, transferrin-binding protein 1 (Tbp1) and transferrin binding protein 2 (Tbp2) also known as TbpA and TbpB respectively, were cloned, expressed and protein products purified. Vaccine formulations containing all three proteins were tested in mice to determine the Th1/Th2 response. These results showed that Tbp2 induced an increase in the production of IFNγ, a cytokine of the cellular immune response. ELISAs and proliferation assays were used to evaluate whether experimentally infected cattle immunized with the three proteins generated both humoral and cellular immune responses. Results suggested that TonB-dependent receptor 1372 initiates both a proliferative response and a humoral response while Tbp1 and Tbp2 induce a cellular and humoral immune response, respectively, in cattle.

This work provides a better understanding of the immune responses generated against *H. somni* infection and aids in the development of a vaccine capable of preventing both respiratory and systemic disease.

ACKNOWLEDGEMENTS

Many important individuals played a large part in the success of this thesis. In particular, I would like to acknowledge my supervisor Dr. Andrew Potter for his constant support throughout my thesis. No matter how busy he was, he always made himself available to provide explanations, support and answer any questions. I especially appreciate the opportunity to explore my love of science and research under his guidance.

I would like to thank Drs. Janet Hill, Volker Gerdts, Wolfgang Koester, and Hugh Townsend for being members of my committee, providing guidance and encouragement, and always making themselves available to help.

I would especially like to thank Neil Rawlyk who was a constant support throughout this project and always willing to explain methods, troubleshoot, and share his expertise. I am forever grateful for all of the things I have learned from Neil and will never forget his kindness and understanding. I would also like to thank the other members of the lab, in particular Gord Crockford and Claudia Avis-Madampage who were always available to answer questions and provide encouragement and support.

I would like to thank the members of Volker Gerdts' and Heather Wilson's labs. They opened their lab to me and were always available to answer questions and provide direction. In particular, I would like to thank Glenn Hamonic who made this thesis possible by teaching me all of the immunological protocols used in this study. A special thanks to fellow graduate students, especially Alyssa Chaffey for her support and help with various analysis and graphing techniques.

A very special thanks to Sam Attah-Poku and Will Deck who are responsible for finding and purifying the very difficult protein, Tbp1. For this I will always be grateful.

Finally, I would like to thank my family especially my parents, Randy and Denyse. I will always appreciate their constant encouragement, loving support, and hugs. I would also like to thank my brothers, especially David who always made me laugh and helped me let go of the occasional stress.

This work was supported by ALMA and Alberta Cattle Feeders Association.

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LIST OF ABBREVIATIONS

ºC	degree Celsius
µg	microgram(s)
µL	microliter(s)
µM	micromolar
AP	alkaline phosphatase
APC	antigen-presenting cell
B cell	B lymphocyte
BCIP	5-Bromo-4-Chloro-3-indolyl phosphate p-toluidine salt
BHI	Brain-heart infusion
BRD	Bovine Respiratory Disease
CFSE	Carboxyfluorescein succinimidyl ester
CFU	colony-forming units
ConA	Concanavalin A
CTLs	cytotoxic T lymphocytes
ddH_2O	double-distilled water
DC	dendritic cell
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fur	Ferric Uptake Regulator

His^6-tag	Histidine tag
HIV	human immunodeficiency virus
IFNγ	Interferon-gamma
IgBPs	immunoglobulin binding proteins
IgG2	immunoglobulin G2
IL-4	Interleukin-4
IL-5	Interleukin-5
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRP	iron-regulated protein
ITME	infectious thrombotic meningoencephalitis
L	litre(s)
LB	Luria-Bertani broth/agar
LktA	Leukotoxin structural gene
LOS	lipooligosaccharides
MBP	Maltose-binding protein
mg	milligram(s)
MHC	major histocompatibility complex
mL	millilitre(s)
mM	millimolar
MPL	monophophoryl lipid A
NBT	Nitrotetrazolium Blue Chloride
ng	nanogram(s)
NK	natural killer

NLR	Nod-like receptor
OD	optical density
PAMP	pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBSA	Phosphate-buffered Saline A
PCR	polymerase chain reaction
PNPP	P-Nitrophenyl phosphate
PRR	pattern-recognition receptor
RO	reverse osmosis
qPCR	quantitative polymerase chain reaction
RPL19	Ribosomal protein L19
rpm	revolutions per minute
SDS	Sodium dodecyl sulfate
Tbps	Transferrin-binding proteins
Tbp1	Transferrin-binding protein 1
Tbp2	Transferrin-binding protein 2
TBS	Tris-buffered saline
TBST	Tris-buffered saline + Tween 20
T cell	T lymphocyte
TLR	Toll-like receptor
TSA	Tryptone Soya Agar
x g	relative centrifugal force

1.0 INTRODUCTION

Histophilus somni (*H. somni*), previously known as *Haemophilus somnus*, is an economically important pathogen. It is a component of the Bovine Respiratory Disease complex and has a large impact on the cattle industry in North America. *H. somni* infection is the leading cause of economic loss in fall-placed feedlot calves in Alberta. Disease symptoms include myocarditis, septicemia, bronchopneumonia, arthritis, pericarditis and pleuritis. This pathogen is able to cause both local and systemic disease with the incidence of systemic infection increasing over the past decade.

Existing vaccines are ineffective at protecting against the systemic form of the disease that includes myocarditis, pleuritis and arthritis. Commercially-available vaccines are composed of killed whole cells using strains isolated decades ago; however, there is one exception that is a bacterial extract vaccine that includes *in-vivo* expressed antigens such as iron-regulated outer membrane proteins. These proteins are TonB-dependent and function as part of the TonB-dependent transport system. This system is common to most Gram-negative organisms and is involved in the acquisition of iron from the host.

I hypothesized that vaccination with these TonB-dependent proteins produced by *H. somni* would be protective against experimental infection and that a good cellular immune response would be induced. Characterization of the immune responses to *H. somni* TonB-dependent proteins would aid in the selection of potential vaccine candidates. These can then be used in the formulation of a vaccine to provide protection against the respiratory and systemic forms of the disease. This could have a significant impact on the cattle industry by lowering the cost of treatment and mortality to cattle producers in North America.

2.0 LITERATURE REVIEW

2.1 Bovine Respiratory Disease

2.1.1 Pathogenesis

Bovine Respiratory Disease (BRD) is the leading cause of death and economic loss to the cattle industry worldwide resulting in estimated losses of $800-900 million per year in the United States [1-5]. It results in 70-80% of morbidity and 40-50% mortality in Unites States' feedlots [5]. BRD is a multifactorial syndrome where a number of agents such as bacteria and viruses as well as the presence of stressors, combine through complex interactions to cause disease [6, 7]. Stressors include weaning, transportation, overcrowding, harsh weather, pregnancy, compromised host immune system and exposure to multiple pathogens. These factors contribute to the occurrence of BRD due to infiltration of bacterial organisms into the lungs following stress or viral infection [1, 7-9]. The disease likely begins when a viral infection weakens the calf's immune system [3, 7]. This infection may result in damage to the respiratory mucosa and modification or suppression of the host immune responses promoting a secondary bacterial infection by the opportunistic pathogens described below resulting in bronchopneumonia [1, 3, 7-10].

2.1.2 Bovine Respiratory Disease Pathogens

H. somni is among the bacterial pathogens associated with BRD [6, 7, 11, 12]. It plays an important role in feedlot disease along with other bacteria such as *Mycoplasma bovis*, *Mannheimia haemolytica* and *Pasteurella multocida*. Many viral agents also play a role in BRD including bovine herpesvirus 1 (BoHV-1), bovine viral diarrhea virus (BVDV), parainfluenza 3 virus (PI3V) and bovine respiratory syncytial virus (BRSV) [1-3, 6, 7]. Due to the multiple factors involved in BRD, agents isolated in individual cases can be quite variable. The most common bacterial pathogen associated with BRD infections is *M. haemolytica* that is often found in mixed infections

with *M. bovis* and BVDV [2]. *Mannheimia haemolytica*, *P. multocida* and *H. somni* are commensal organisms of the upper respiratory tract and become pathogens in the lungs during suppression of the immune system due to previously mentioned stressors [2, 7]. There are many studies that examine each pathogen, however it is still often difficult to identify the causative agent due to the complexity and variability of BRD and it continues to be a major cause of financial loss to the cattle industry [3, 7, 13, 14].

2.1.3 Economic Impact

BRD is an important cause of economic loss in the dairy and beef cattle industries [3, 6, 7, 15, 16]. The costs of BRD medical treatment are substantial and have an impact on the carcass merit and meat quality increasing losses due to reduced meat yield and increased mortality [2, 4]. In the United States, BRD has been estimated to cost the livestock industry $800 to $900 million per year due to death, reduced feed efficiency and treatment costs [4]. Specific losses per treated calf have been estimated at $23.60 [7]. There have been recent technological, biological and pharmacological advances to fight BRD including vaccines that are important in preventing economic loss however, BRD continues to be a burden on the cattle industry [6].

2.1.4 Control of Bovine Respiratory Disease

There are vaccines available that target different viral and bacterial agents associated with BRD. Commercially available vaccines for BRD commonly include BVDV, BoHV-1, PI3V and BRSV [17]. Unfortunately, vaccination has shown inconsistent efficacy against some bacterial agents such as *M. haemolytica*, *P. multocida*, and *H. somni* [2, 7]. This inconsistent efficacy has resulted in the use of antimicrobials as the primary source of management. Antimicrobials can be effective in reducing the incidence of BRD in large feedlots that explains why 20-50% of new arrivals in western Canada receive injectable metaphylactic antimicrobials as a preventative

measure [2]. This antimicrobial use raises concerns of driving the evolution, persistence and spread of antimicrobial resistance in livestock pathogens as well as commensal organisms [2]. If widespread resistance were to occur in BRD pathogens, the economic losses to the cattle industry would become very severe [2].

There are management factors that can increase the risk of BRD. For example, calves purchased from sale barns will have a higher risk of developing BRD due to the increase in transportation, marketing and mingling with animals from other farms [13]. The introduction of purchased calves involves activities such as dehorning, castrating, administration of vaccines, growth promoting implants and abortion of any pregnant heifers which can all result in an increase in stress [13, 14]. Since there has been no success in finding processing practices that eliminate BRD, preconditioning measures have been identified for pre-shipment of calves. Common components of preconditioning include: vaccination for respiratory viruses and sometimes bacteria such as *M. haemolytica*, *P. multocida* and *H. somni*; dehorning and castration with adequate healing time; weaning prior to shipment; training calves to eat and drink from troughs. The goal is to eliminate stressors happening over the course of days and instead allowing days or weeks for the calves to adjust [14]. Unfortunately, the results of preconditioning studies are difficult to interpret because on-farm morbidity during preconditioning is often not reported. Therefore, BRD may not be reduced and instead the disease is postponed to occur with the cow-calf producer [14].

2.2 General Immunology

2.2.1 Innate Immune Response

Innate immunity is the first line of defense and it recognizes foreign material such as the nucleic acids, carbohydrates and proteins of pathogens that are not produced by the host. This recognition of foreign material is dependent on pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin-like receptors and cytosylic Nod-like receptors that are capable of sensing a wide range of microbial stimuli such as pathogen-associated molecular patterns (PAMPs) [18, 19]. Innate immunity consists of elements that are always present such as the skin and mucous membranes that present physical barriers and chemical components such as pH, fatty acids and antimicrobial peptides [20]. These components, that are always present, enable the innate immune response to respond quickly and efficiently due to the presence of PRRs on the epithelial cells and innate cells circulating the body [21]. Other components of innate immunity include phagocytic cells such as neutrophils and macrophages, dendritic cells (DC), natural killer (NK) cells and serum proteins such as the components of the complement system, β-lysin, lysozyme and mediators of inflammation [19, 22]. All of these elements participate in either the destruction of pathogens or in increasing the effectiveness of host reactions by inducing adaptive immunity [20].

Histophilus somni can be found living as a commensal organism in the upper respiratory tract of cattle. During stress such as viral infection, transportation or weaning *H. somni* causes disease by adhering to nasal and respiratory epithelial cells [23]. How this pathogen adheres to epithelial cells is not yet defined, however it may involve heparin-binding motifs. Heparan sulfate proteoglycans coat the endothelial cells and form a charged layer that plays a role in anticoagulation [23]. *Histophilus influenzae* is known to adhere to human respiratory epithelial

cells, cause focal damage to respiratory mucosa and adhere to damaged cells [23]. Evidence suggests a connection between increased adherence and cell damage but there is no evidence of damage to the epithelial cells during *H. somni* infection. Perhaps damage is caused by other pathogens such as the viral components of BRD previously discussed [23]. Interaction with epithelial cells results in the stimulation of PRRs present on epithelial cells and innate cells such as neutrophils and macrophages [24]. These receptors sense microorganisms by recognition of PAMPs such as microbial proteins, lipids, carbohydrates and nucleic acids [24]. Recognition leads to the production of inflammatory cytokines, chemokines and type I interferons. Many types of PRRs exist including the well-studied TLRs mentioned previously, which are capable of detecting microbial nucleic acids, lipoproteins, lipopolysaccharide and flagellin [24, 25]. These receptors modulate the immune responses including cytokine signaling and secretion, phagosome maturation, nod-like receptor (NLR) activation and autophagy. Another important PRR is the NLR that detects bacterial molecules in the cytoplasm of cells, works synergistically with TLR activation and triggers inflammatory cytokine secretion [24, 25]. In order to cause systemic disease, *H. somni* must be capable of crossing the epithelial barriers that separate the inner and outer lung however, how is unknown. Many possibilities exist such as direct transmigration, the use of infected leukocytes as a Trojan horse, induction of apoptosis or necrosis, coagulation that increases vascular permeability, and other mechanisms that result in compromised function of the epithelium [23].

In order to infect leukocytes and possibly travel systemically via these infected cells, *H. somni* must be capable of surviving intracellularly. While acting as antigen presenting cells for activation of lymphocytes, macrophages patrol the body and engulf pathogens into a vacuole where they are able to direct high concentrations of reactive oxygen species, reactive nitrogen

intermediates and antimicrobial peptides [25]. There is evidence that *H. somni* can survive and replicate to an extent in bovine macrophages through the inhibition of reactive oxygen and nitrogen intermediates. It has also been shown that neutrophils that ingest *H. somni* have compromised bactericidal activity and change in a way that is similar to apoptosis [23]. Neutrophils produce neutrophil extracellular traps (NETs) and while it is undefined how *H. somni* triggers this reaction, these traps are produced during an *H. somni* infection much like *N. meningitidis* and *H. influenzae*. The release of NETs is induced by activated platelets, which bind the neutrophil. The neutrophil then releases decondensed chromatin that forms a sticky mesh decorated with antimicrobial peptides such as histones, elastase, cathepsin G and myeloperoxidase [23, 26]. These traps ensnare pathogens like *H. somni* in the vasculature and help to limit dissemination [26].

H. somni is capable of adhering to epithelial cells but it can also adhere to endothelial cells that line the blood vessels. Adherence to these cells results in the induction of inflammatory cytokine tumor necrosis factor-alpha (TNF-α), which also results in increased adherence [23]. *H. somni* infection often results in the formation of thrombosis, a tightly regulated reaction by a system of anticoagulant mechanisms that ensure homeostasis. There are three steps to initiation of coagulation and thrombis formation [27, 28]. When endothelial cells are injured, not functioning or disrupted, tissue factor is exposed to factor VIIa found in the blood and this is the initiation step. Factor VIIa and tissue factor form a complex that initiates coagulation by activation of factors IX and X, which generate factor IIa also known as thrombin. The second step, amplification, is the rapid production of thrombin to form a stable clot. This leads to the third step, propagation, where recruitment of activated platelets to the site of injury takes place. These platelets provide the mediators for optimal generation of thrombin that leads to the production of fibrin from fibrinogen to make a stable blood clot [27, 28]. Many of these innate responses that are induced during *H.*

somni infection result in the activation of the adaptive immune response. For example, innate cells such as neutrophils, dendritic cells and macrophages will continue to patrol the body and act as APCs to lymphocytes by presenting antigenic peptides in the lymphnodes. The stimulation of cytokines and chemokines also induces the pathogen-specific and long-lasting memory provided by B and T cells of the adaptive immune response [26-28].

2.2.2 Adaptive Immune Response

The adaptive immune response requires stimulation by exposure to a pathogenic microbe and its magnitude and capabilities increase with each exposure to a particular antigen [21]. This means that the adaptive immune response is more specific than innate and involves memory [20, 21]. The components of adaptive immunity include but are not limited to lymphocytes, secretion of antibodies, secretion of cytokines that regulate and coordinate the activities of both the innate and adaptive immune cells and chemokines that regulate cell migration and movement to tissue sites [20, 21]. There are several types of immunity including Th1, Th2, Th3, Th17 and others. However, the two most important types of adaptive immunity are cell-mediated and humoral immunity or Th1 and Th2, respectively [20, 21, 29]. T cells mediate cell-mediated immunity, which is required to combat intracellular microbes, such as *H. somni*, as they are inaccessible to circulating antibodies [21]. T lymphocytes promote killing of infected cells, cooperate with B cells to enhance production of effective antibodies, induce inflammatory responses and regulate immune responses [20, 21]. Antibodies present in blood and mucosal secretions mediate humoral immunity [20, 21]. The antibodies recognize microbial antigens or their toxins and neutralize or mark them as targets for elimination. Antibodies are also capable of activating mechanisms such as phagocytosis and the release of inflammatory mediators while also providing defense to mucosal surfaces and newborns [21].

When adaptive immunity is involved in controlling an infection, APCs such as dendritic cells and macrophages are located in the epithelia and connective tissues where they capture microbes, digest proteins and move to the lymph nodes to present antigens to lymphocytes [21, 22]. The digested protein fragments are expressed via either class I or class II major histocompatibility complex (MHC) molecules on the surface of the cell and are presented to naïve T cells in the lymph nodes [29]. T cells can be functionally divided into helper T cells and cytotoxic T cells, or $CD4^+$ and $CD8^+$ respectively. Both helper T cells and cytotoxic T cells are MHC restricted as $CD4^+$ T cells recognize antigenic peptides presented on MHC class II while $CD8^+$ T cells recognize peptides presented on MHC class I [29]. The differentiation of the adaptive immune response towards a Th1 cell-mediated response or a Th2 humoral response depends on the activation of helper T cells. The activation of T cells requires three signals. The first signal is the binding of the T-cell receptor (TCR) to an antigen-MHC complex. Costimulatory molecules provide the second signal and cytokines provide the third [29, 31]. Costimulatory molecules include but are not limited to CD28, OX40, ICOS and TIM-1 [29]. Upon activation, helper T cells differentiate into effector and memory T cells that produce and secret cytokines that drive the proliferation of T helper cells, activate B cells and induce the differentiation of cytotoxic T cells into CTLs that recognize and lyse infected self-cells [29].

Cytokines are molecules produced during the innate immune response and are important costimulators. The major cytokines secreted by PAMP-stimulated cells act on both B and T lymphocytes and include IL-12, IL-1, IL-6, IL-23, IL-15 and IL-6. The cytokine IL-12 stimulates the $CD4^+$ T cells to differentiate into Th1 effector cells. Cytokines IL-1, IL-6 and IL-23 stimulate them to differentiate into Th17 helper cells [21]. T lymphocytes that are $CD8^+$ differentiate into cytotoxic T lymphocytes (CTLs) that patrol the body and recognize and lyse infected host-cells.

Survival and memory of these $CD8^+$ T cells, which are both important for immunity and long-term infection, are promoted by IL-15. [20-22]. These CTLs are involved in the cellular or Th1 response and are key in combatting intracellular pathogens [18, 29].

Differentiated B cells result in the production and secretion of different classes of antibodies, promoted by IL-6, that are involved in the humoral or Th2 response. Different classes of antibodies perform specialized functions that are each important in humoral immunity. These classes include the isotypes IgA, IgE, IgM and IgG [21]. Isotype IgA plays a primary role at mucosal surfaces where it is actively secreted across the epithelial barrier. IgE is primarily involved in anti-parasitic and hypersensitivity reactions while IgM is a serum-antibody and a naïve B cell antigen receptor. IgG is the primary serum-antibody involved in opsonisation, activation of the complement cascade and neonatal [21].

Passive immunization is a method of transferring antibodies from one individual to another. It has been shown that calves can be protected from *H. somni* through passive immunization [23]. More specifically, IgG2 appears to be more protective than IgG1, IgM and IgA as the levels of IgG2 peak later and last longer post-infection [23, 30]. IgA is thought to be important for mucosal surfaces and shows an increase during *H. somni* infection however, as IgG2 levels increase, IgA levels as well as the levels of *H. somni* decrease. Perhaps IgA is blocking epitopes that are important for IgG2 opsonization and this limits the protection that is provided by opsonisation of *H. somni* bacteria by IgG2 [23]. *H. somni* has proven capable of evading these immune responses through mechanisms such as interfering with phagocyte function, shedding of antigens that bind antibodies, decreased immune stimulation and antigenic variation, allowing infection to persist [30].

It is possible to analyze Th1 and Th2 responses through the detection of specific cytokines secreted by cells involved in each response. For example, Th1 responses can be determined by analyzing Interferon-gamma (IFN γ) or Interleukin-2 (IL-2) levels, while Th2 responses can be determined by analyzing levels of Interleukin-4 (IL-4) or Interleukin-5 (IL-5) (Refer to Figure 2-1) [29].

2.2.3 Vaccines and Adjuvants

Vaccines have been used for centuries and offer an effective and cost-efficient means of providing protection against infection [18, 19, 31]. Vaccines are killed or attenuated forms of an infectious agent that is administered to healthy individuals to elicit an immune response [19, 21]. This immune response provides the individual with protection against the live, pathogenic microbe [21]. As described above, the innate immune system is a key component in sensing foreign substances such as vaccines and adjuvants that stimulate the development of a protective response [18]. There are many types of vaccines, including attenuated, inactivated vaccines, subunit vaccines, DNA vaccines and recombinant vaccines [21]. Live attenuated vaccines are composed of weakened versions of virus or bacteria that induce immunity similar to the protection gained from a natural infection [18, 21]. Inactivated vaccines contain killed pathogens and often require adjuvants in order to elicit a strong enough immune response [31]. The subunit vaccines can include purified antigens, toxins and carbohydrates along with adjuvants, which enhance and modify the immune response induced. Some subunit vaccines contain bacterial polysaccharides coupled to proteins to form a conjugate vaccine, which elicits a high-affinity antibody response [18, 21, 31]. Most vaccines induce the production of neutralizing antibodies. These antibodies are useful in neutralizing pathogens such as toxin-secreting bacteria and their toxins, blood-borne

viruses and mucosal viruses and can be measured with assays that measure functional antibody activity such as an enzyme-linked immunosorbent assay (ELISA) [18].

Adjuvants were first described in 1924 as "*substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone*" (Ramon, 1924) [31, 32]. Adjuvants are used because they stimulate a stronger, faster and longer lasting immune response. They also allow the modification or directing of the immune response as they can be used to specifically enhance either the antibody or the cellular immune response [31]. Adjuvants can be composed of many different substances including but not limited to oils, virosomes, TLRs, monophosphoryl lipid A (MPL), unmethylated CpG dinucleotides and heat-killed bacteria [19, 21]. A mineral salt adjuvant, Alum is the oldest adjuvant and has been used successfully in animal and human vaccines since the 1940s [19, 31, 32]. Adjuvants can help vaccines become more immunogenic in many ways. They can induce a protective immune response earlier, which is important during an outbreak. They can reduce the number of immunizations as well as the amount of vaccine material necessary. Adjuvants can also elicit protection in the young and the elderly and allow vaccination through new routes such as mucosal membranes [31]. The mechanisms of action of adjuvants are not completely understood however, it is known that they are capable of using one or more mechanisms. Some of these mechanisms include acting as a signal of danger, forming a depot at the site of infection and activation and maturation of APCs [31, 32]. The adjuvant is recognized by the PRRs present on APCs and this recognition informs the immune system that there is an ongoing event or danger present and as a result, the innate immune response is up regulated. Subsequent activation of the adaptive immune response is also initiated however; the type of adaptive response mounted depends on the danger signal [31]. When the adjuvant forms a depot at the site of injection, the antigen is absorbed to the adjuvant and this allows for the slow-

release of antigen to ensure constant stimulation of the innate immune response [31, 32]. As the APCs arrive at the site of injection, they take up the vaccine, process, and present peptides to helper and cytotoxic T cells. As mentioned above, activation of these T cells requires the presence of a costimulatory molecule which depends on the danger signal itself. Therefore, adjuvants are capable of influencing the type of immune response that is induced [31, 32].

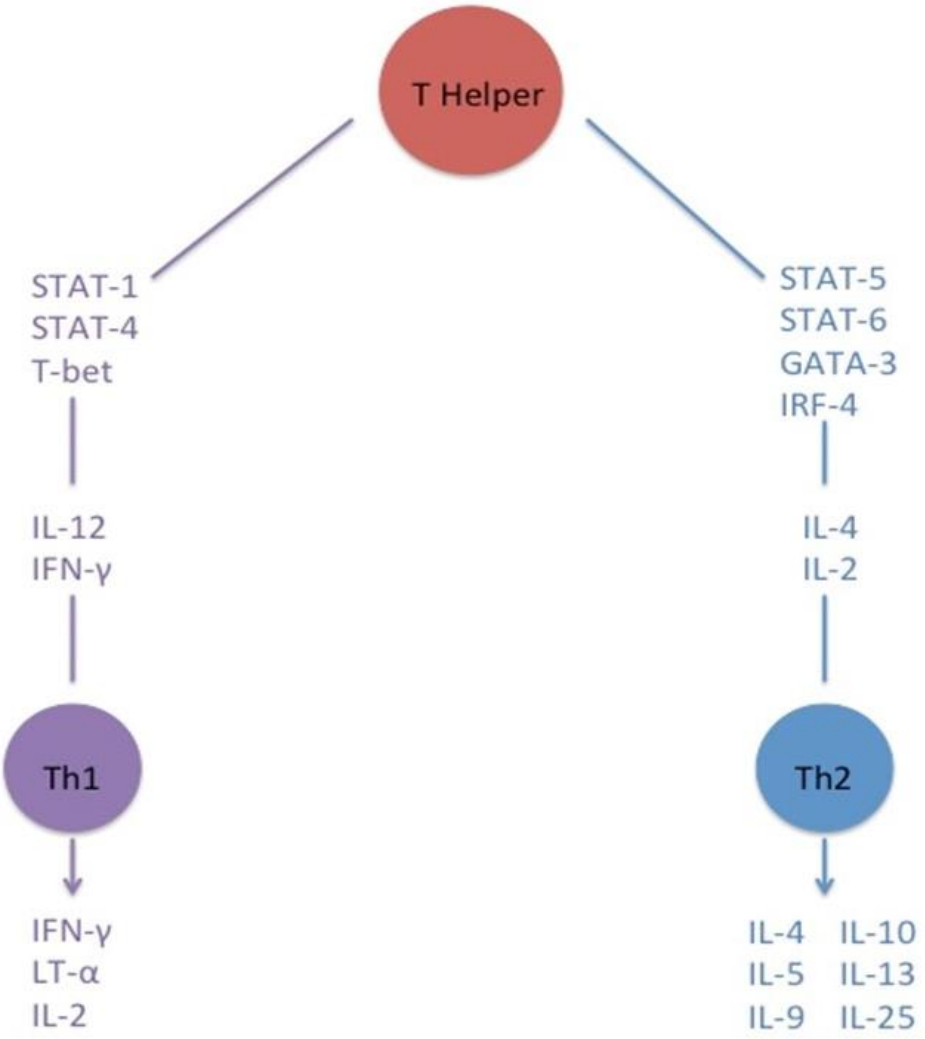


Figure 2-1 Summary of Th1 and Th2 immune response development. Transcription factors T-bet and GATA-3 are essential for the development of both Th1 and Th2 responses, respectively. Cytokines are released directing T-helper cell differentiation. Cytokines secreted by Th1 cells are IFNγ and IL-2, while cytokines secreted from Th2 cells are IL-4 and IL-5. Adapted from Abdoli *et al.* 2015.

2.3 *Histophilus somni*

2.3.1 Characteristics and Significance

Histophilus somni is a Gram-negative coccobacillus, non-encapsulated and non-motile member of the *Pasterellaceae* family. It does not have pili or flagella and does not form spores [3, 6, 9, 12, 33-35]. *H. somni* is an intracellular pathogen that shows strict host-specificity in ruminants, most notably in cattle [6, 36]. The disease has a large economic impact on the livestock industry but more specifically the cattle industry [34, 35]. *H. somni* is described as an opportunistic and facultative intracellular pathogen that under normal circumstances is relatively non-invasive [6, 10, 12, 33, 37]. It is a part of the microflora of the lower reproductive tract as well as the mucosal surfaces of the upper respiratory tract of cattle and other ruminants [3, 6, 9, 12, 16, 33]. During the presence of stressors such as overcrowding, viral infection and transportation, *H. somni* is capable of causing disease [9, 12]. Identification of *H. somni* can be difficult due to its fastidious growth conditions as well as the fact that it is often found alongside other bacterial pathogens such as *M. haemolytica*, *P. multocida*, or *T. pyogenes* [2, 36]. Often, these other organisms result in *H. somni* not being isolated from culture due to their overgrowth [2].

2.3.2 Virulence Factors

H. somni has a variety of virulence factors that have been explored since its first isolation in 1956 [9, 16, 34, 36]. These include adherence factors, phase variation of surface proteins, lipopolysaccharide (LPS) and lipooligosaccharides (LOS), outer membrane proteins, serum resistance, biofilm formation and iron-binding proteins [3, 9, 12, 34-36, 38]. These *H. somni* virulence factors allow it to protect itself from host defences, induce inflammation, damage and evade the immune system and promote colonization of various tissue sites [9, 38]. The virulence factors cause many negative effects on the host, such as apoptosis of bovine endothelial cells,

platelet aggregation, pro-inflammatory response, intracellular survival and activation leading to endothelial cell death [3, 9, 16]. *H. somni* also produces proteins capable of binding the Fc region of immunoglobulin [9, 12, 38, 39]. These proteins known as immunoglobulin binding proteins (IgBPs) are able to bind to antibodies such as Immunoglobulin G2 (IgG2) [3, 9, 12, 34, 35, 39-41]. The non-specific binding of these proteins to antibodies prevents antibody-mediated inactivation of *H. somni* [9, 35]. Transferrin-binding proteins (Tbps) have also been identified in *H. somni*. These proteins are responsible for scavenging iron from the host and may be another factor effecting virulence and host immunity [9, 12, 35, 38]. The acquisition of iron and other nutrients, attachment to host cells, and interactions with phagocytic cells are among *H. somni*'s other virulence-associated factors that result in the modification of phagocytic cell function and therefore the modification of host immunity [34].

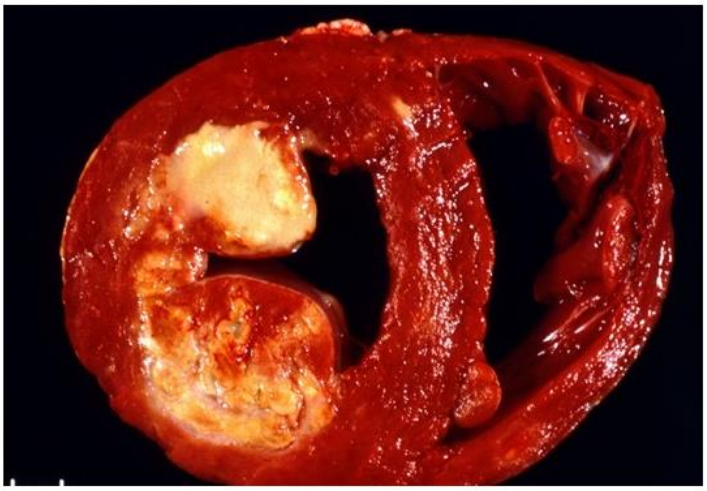
2.3.3 Pathogenesis

H. somni infection is represented by various symptoms such as septicemia, infectious thrombotic meningoencephalitis (ITME), polyarthritis, myocarditis, bronchopneumonia, pneumonia, and pleuritis [3, 12, 15, 33-35, 38, 40-45]. As mentioned above, bacterial colonization occurs after a viral infection suppresses the immune system and *H. somni* moves into the lower respiratory tract [1, 3, 7-10, 30]. *H. somni* is capable of colonizing the surfaces of mucosal membranes and attaching to non-epithelial cells [36]. It protects itself in host tissues, especially during colonization of the heart through the formation of a biofilm. This biofilm protects the organism from host immune responses and antibiotics [34]. *H. somni* is able to gain entry into the bloodstream where it is able to internalize in immune cells and disseminate to various areas of the body such as the brain, heart, skeletal muscle, joints, kidneys and liver [36, 42, 45]. Septicemia and localization in the heart and joints has occurred with increased frequency since the late 1980s.

The occurrence of ITME is less frequent while more myocarditis and arthritis are reported [42, 44]. This may be due to the adaptation of *H. somni* to the increased use of vaccines and mass treatment with antibiotics [42, 44, 45]. However, strains with different virulence factors may have evolved through natural selection and while the organism changed, the immune responses of the host population varied [42].

Myocarditis occurs more in yearlings than in weaned calves who appear either chronically ill or perfectly healthy before they die suddenly [42, 45]. The cause of death is acute heart failure accompanied by evidence of pulmonary congestion and edema [42]. Localization in the heart results in infarction, inflammation and lesion formation (Figure 2-2A) resulting in cardiac failure and death [45]. Arthritis is also a major symptom of current *H. somni* infection. Multiple joints are affected (Figure 2-2B) and the animals show signs of stiffness, lameness, and knuckling at the fetlocks. At necropsy, affected joints show obvious infection through excessive fluid accumulation and the presence of fibrinous clots [42].

A



B

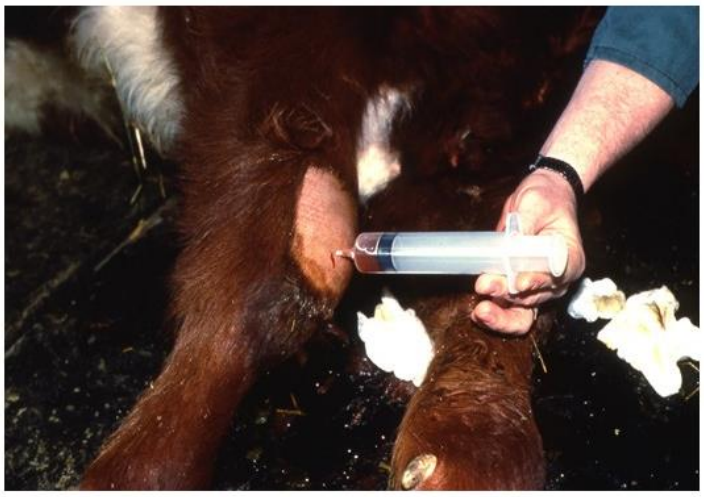


Figure 2-2: Myocarditis and arthritis resulting from *H. somni* infection. (A) The myocardial lesions formed during infection. (B) Arthritis with swelling and fluid-filled joint.

H. somni shows resistance to intracellular killing, observed by an abundance of bacteria in phagocytic cells, such as macrophages and neutrophils, within diseased organs. Neutrophils are able to internalize the bacteria but are ineffective at killing it as the cells remain viable within the vacuole [9, 36]. It has been shown that neutrophils isolated from cattle infected with *H. somni* have exhibited decreased anti-bacterial activity [30]. By surviving within phagocytic cells, *H. somni* is capable of avoiding other host defenses and invading various tissues, which likely contributes to the development of a chronic, multisystemic infection [36]. Incubation of *H. somni* with alveolar macrophages, blood monocytes and neutrophils decreases their ability to phagocytose as well as their ability to produce oxygen-free radicals and kill bacteria [16]. When cultured with bovine blood monocytes, *H. somni* has the ability to survive and multiply even when the immune cells are treated with cytokines, which demonstrates *H. somni*'s ability to avoid killing by macrophages [37]. These infected macrophages serve as a depot for infection that may explain in part, the subacute to chronic course of the infection [37]. Survival within mononuclear phagocytes is a strategy that many other pathogens have developed, including Mycobacteria, *Brucella*, *Salmonella*, *Yersinia* and *Listeria* [37]. By surviving within phagocytes, *H. somni* is able to protect itself from the immune system, spread throughout the body and cause both local and systemic disease [8, 10, 11, 16, 36, 37].

2.3.4 Immunity to *H. somni* infection

The interaction of *H. somni* with the host innate immune system begins with evading the neutrophils. As described above, *H. somni* is adept at avoiding neutrophil killing and it has been discovered that *H. somni* is capable of altering neutrophil activity once ingested [30]. Mononuclear phagocytes also show limited ability to kill *H. somni*, which appears capable of surviving and replicating within bovine monocyte-derived and alveolar macrophages. *H. somni*, both in its free

form as well as intracellular form, will interact with the endothelium of the blood vessel walls. *H. somni* adheres to the endothelial cells and activates nearby cells that release the inflammatory cytokine tumor necrosis factor alpha (TNF-α) [30].

The methods of controlling *H. somni* infection are vaccination, mass treatment with antibiotics, and vaccination against other BRD pathogens [30]. There are commercially available vaccines that specifically target *H. somni* however; they were developed from strains isolated in the 1970s. These vaccines were developed to address the nervous system form of the disease, ITME. ITME was the only clinical form of the disease recognized during the 1970s [9, 10, 42]. Since then other disease conditions were becoming more common and new manifestations of the disease were being reported [42]. During the 1990s, haemophilosis was recognized as a significant cause of mortality, along with myocarditis and arthritis, in fall-placed feedlot calves in western Canada [44].

There are 26 USDA-approved vaccines developed for the American market that target *H. somni* and are heavily used however, their ability to provide a protective immune response is modest or unknown [30]. Many currently available vaccines utilize killed whole cell bacterins that have been relatively successful in reducing the risk of ITME [35]. These whole cell vaccines however, do have a reputation for occasionally causing severe side effects and do not provide protection, which is why the development of new vaccines is necessary [41]. Subunit vaccines provide a way around the problems often caused by killed whole cells. One such vaccine is the currently available Somnu-Star PH (Elanco Animal Health), an acellular vaccine containing *in-vivo* expressed proteins. These proteins include iron-regulated outer membrane proteins, obtained from cultures of *H. somni*, which aid in reducing inducing a protective immune response against BRD [35, 46, 47]. It is possible that these currently available vaccines are not effective against *H.*

somni when it is in a biofilm but only protects against planktonic cells, another reason more research and vaccine trials are necessary [34].

2.4 TonB-dependent Proteins

2.4.1 TonB-dependent Transport System

Iron is an essential nutrient for all organisms but it is not readily available under normal conditions in the host [48-51]. Iron is a component of erythrocytes and bound to haemoglobin, hemopexin or haem. Since iron-binding proteins such as haemoglobin, transferrins, lactoferrins and ferritins chelate iron, it is not readily available to pathogens [49, 52-54]. Transferrins have an N and a C lobe that are each able to bind ferric iron and sequester it from pathogenic organisms that have developed countermeasures to acquire iron [52, 54]. Iron is important to microorganisms since it is a key component of important metabolically-linked compounds so acquisition during infection is imperative to survival and replication of pathogens [49].

Bacteria have developed many strategies that allow them to obtain iron from their host. Many bacteria produce low-molecular-weight iron binding ligands that have a high affinity for iron while others are able to utilize haem-bound iron. *Serratia marcescens* uses a haem-binding protein called a haemophore that is secreted and later taken up through the use of receptors [49]. Another strategy bacteria have developed involves the TonB-dependent transport system common to most Gram-negative organisms [50]. This transport system is regulated by the supply of iron, as high levels of iron can be toxic to the cell. In *E. coli*, the Ferric Uptake Regulator (Fur) regulates the expression of genes involved in iron such as transferrin-binding proteins (TBPs). When iron levels are high, the Fur repressor binds the DNA sequence called the Fur box using Fe^{2+} as a cofactor. The binding of the Fur repressor to the box represses transcription of genes involved in the uptake of iron [50]. During iron-depleted conditions, the TonB-dependent outer membrane

proteins are synthesized in large amounts and their activity increases [51]. Iron is acquired by either direct binding to bacterial outer membrane proteins or by the release of siderophores that acquire iron from host proteins and bring it back to specific TonB-dependent receptors [48, 49, 52]. The TonB-dependent complex includes three inner membrane proteins TonB, ExbB and ExbD that couple the energy from the proton motive force in the inner membrane to the outer-membrane [48-51]. This energy allows the outer membrane proteins to actively pump siderophores into the periplasm [48]. There are many other key players involved in the TonB-dependent transport system, as it is a complicated process. It is energy expensive and requires energy derived from a proton motive force across the inner membrane [50].

2.4.2 TonB-dependent receptor, Tbp1 and Tbp2

Most Gram-negative bacteria have TonB-dependent transporters involved in the acquisition of various important substrates such as iron, vitamin B_{12}, nickel, and carbohydrates [50]. The number of these transporters present in each organism varies. For example *E. coli* produces 7, *Pseudomonas aeruginosa* produces 34 and *Caulobacter crescentus* makes 65 different TonB-dependent transporters, whose regulation is not yet known [50]. In *H. somni*, the TonB-dependent receptor is a heme/hemoglobin receptor family protein along with three other members of the group. Tbp1 also known as TbpA is a lactoferrin and transferrin receptor protein while Tbp2 also known as TbpB is a transferrin-binding protein [34].

Members of the families *Neisseriaceae, Moraxellaceae* and *Pasterellaceae* such as *Pasteurella multocida, Haemophilus influenzae, Neisseria gonnorrhoeae, Actinobacillus pleuropneumoniae* and *Neisseria meningitidis* use a highly specific uptake system to acquire iron from the host transferrin [52-63]. The components of this uptake system include two transferrin receptors, TbpA and TbpB; a periplasmic protein dependent transport system including FbpA,

FbpB and FbpC; and the third component is the energy transduction system consisting of TonB, ExbB and ExbD [58]. TbpA and TbpB are located on the cell surface of the pathogen and interact directly with serum transferrin (Figure 2-3) [50, 52]. The primary component of bacterial transferrin and lactoferrin receptors is TbpA. This transmembrane protein facilitates the acquisition and relocation of iron into the bacterial periplasm [54]. The cofactor, TbpB, is an accessory lipoprotein that extends from the outer membrane surface using an N-terminal anchor peptide. This lipoprotein is involved in the capture of host transferrin through its binding to the C lobe and may aid TbpA in the removal of iron from transferrin [54, 56]. Both proteins work together in order to bind transferrin, remove the iron and import it across the outer membrane into the periplasm. However, while TbpA is necessary for iron acquisition, cells missing TbpB can still survive in transferrin rich environments, suggesting that TbpA is capable of capturing, removing and relocating iron itself [56-60]. TbpA is highly conserved in *Neisseria* strains while antigenic and sequence variation of TbpB tends to be more widespread however, neither TbpA nor TbpB are subject to high-frequency variation [56-60]. Both TbpA and TbpB are transcribed from the operon *tbpAB*, which is regulated by the regulatory protein Fur, described above [56-60]. It has been shown that *Neisseria gonnorrhoeae* deficient in transferrin-receptors is avirulent during a human gonococcal infection. This supports the idea that transferrin-binding proteins from *Pasteurellaceae* and *Neisseriaceae* play a key role in pathogen virulence [63]. Purified TbpA and TbpB from *Neisseria* are antigenic when used in the immunization of animals and the transferrin receptor system is found in 100% of *Neisseria gonorrhoeae* clinical isolates. This along with the fact that it is not subject to phase or antigenic variation and appears to be necessary in *Neisseria* infection in humans, makes proteins from the transferrin receptor system ideal vaccine candidates [56, 59].

The structures of TbpA and TbpB have been studied in both *Neisseria* and *M. hemolytica* species and while structures have been identified, the exact functions and interactions are still not fully understood. TbpA is thought to be made up of features including long extracellular loops that are involved in the interaction with transferrin, a helix finger that may be involved in the release of iron and a plug domain loop that may be a sensor for ligand binding [56-59]. This structure forms a pore in the outer membrane where iron can pass through into the periplasm [58]. When TbpA is bound to transferrin, the C-lobe of the transferrin appears to be partially open via the separation of C1 and C2 domains. The α-helix and N-terminal plug domain of TbpA are inserted between the separated C1 and C2 domains of the C-lobe. This suggests that the α-helix and plug domain are involved in the removal of iron from the transferrin [56]. TbpB is a surface-exposed lipoprotein that appears to increase the efficiency of iron acquisition by binding to transferrin and saturating it on the cell surface. It also appears to aid in the release of transferrin once iron has been removed and it may also participate in the extraction of iron from transferrin [56, 57, 59]. The binding of TbpB to transferrin is different from the binding of TbpA in that the transferrin is fully closed. This explains the strong preference TbpB has for binding to iron-bound transferrin [56]. Currently, there is a model of iron-transport which involves the binding of transferrin to TbpB followed by TbpB bringing the transferrin to TbpA. Once TbpA binds the transferrin, iron is removed and imported into the cell (Figure 2-3) [59]. Evidence has suggested that antibodies to the loop of TbpA, a presumed key component of the protein, interrupt protein function. Cash *et al.* demonstrated that loop-specific antibodies lead to modest inhibition of TbpA function [57].

Vaccines have been developed to test the antigenicity of Tbps from both *M. haemolyitca* and *Actinobacillus pleuropnaumoniae*. Rossi-Campos *et al*. showed that vaccination with a 60 kDa transferrin-binding protein from *Actinobacillus pleuropnaumoniae* serotype 7 resulted in an

increase in antibody titres, less extensive lung damage and significantly reduced mortality in pigs. They also showed that this transferrin-binding protein was not protective against other *A. pleuropneumonia* serotypes, which was expected as serotypes 1 and 7 were known to express different 60 kDa proteins [64]. Vaccines against *M. haemolytica* have often been bacterins but they were found to have no beneficial effect, a detrimental effect or their efficacy was simply questioned [65]. Once it was realized that the protein produced during logarithmic growth, leukotoxin (Lkt), was important in the pathogenesis of *M. haemolytica*, the production of subunit vaccines began in earnest [65]. Many other antigens were identified and incorporated into subunit or subunit-enriched vaccines, however the most important for this study were the proteins expressed in iron-limiting environments [65]. During iron-restricted conditions, *M. haemolytica* is known to produce iron-regulated outer membrane proteins (IRP) that are absent or weakly expressed in iron-rich conditions [55, 66, 67]. In the 1990s, it was documented that sera from recovered, experimentally infected, sheep contained antibodies specific for these IRPs [67]. The presence of specific antibodies suggested these were protective antigens [67]. Since then, inactivated vaccines containing these IRPs have been more frequently developed to combat BRD and shown to provide protection six weeks after the first immunization [68]. A study by Potter *et al*. looked at the effect on antibody titres when calves were immunized with *M. haemolytica*'s TbpB and TbpA [63]. Calves that received TbpA showed an insignificant increase in antibody titres, while calves immunized with TbpB showed a signficant increase in antibody titres specific for TbpB. When calves were immunized with both proteins, there was no evidence of a significant TbpA-specific antibody response however, TbpA did aid in the overall health of the calves possibly by inducing a cell-mediated immune response [63]. Potter *et al*. also studied the effects of immunization with *H. somni*'s Tbp1 and Tbp2. Here, it was shown that calves immunized with recombinant Tbp2

responded with an increase in antibody titre while their response to Tbp1 was minimal, which is consistent with other studies of Tbp1/TbpA from other organisms [69].

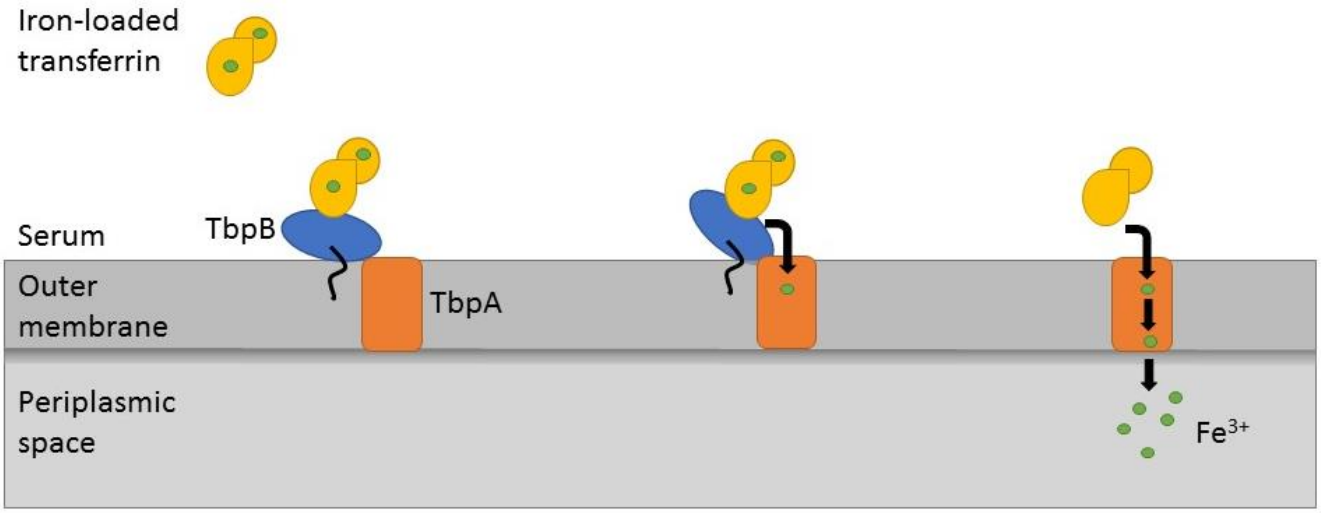


Figure 2-3: The interaction between transferrin-bound iron and the bacterial countermeasures TbpA and TbpB. Pathogens *H. inlfuenzae* and *N. gonnorrhoeae* use receptor TbpA and its co-receptor TbpB to acquire iron during infection. These receptors transport the captured iron to the bacterial periplasm from the host serum. Adapted from Armitage and Drakesmith 2014.

3.0 HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

Understanding the immune responses generated as well as the antigens expressed during infection is essential in producing effective vaccines. TonB-dependent proteins have been shown to be protective in other species in the genus *Pastuerellaceae* therefore it was hypothesized that vaccination with TonB-dependent proteins produced by *H. somni* will be protective against experimental infection and that a good cellular immune response is important for protection.

3.2 Objectives

The objectives of this study were to examine the effects of vaccination with receptor 1372, transferrin-binding protein 1 (Tbp1) and transferrin-binding protein 2 (Tbp2) on the humoral and cellular immune responses of cattle during experimental infection with *H. somni*. The goals of this study were to: (1) Clone and express the genes coding for TonB-dependent proteins of *H. somni*, (2) determine whether or not experimentally-infected cattle generate both humoral and cellular immune reponses against TonB-dependent antigens, (3) formulate vaccines capable of generating balanced Th1/Th2 responses and confirm in mice, and finally to (4) vaccinate cattle and characterize the immune responses toward and protective capacity of the antigens.

4.0 MATERIALS AND METHODS

4.1 Bacterial strains, growth conditions and plasmids

The *E.coli* strains used in this study were DH5αF'IQ [35] and M15 [35] provided by the laboratory collection, BL21Plys [70] provided by the lab of Dr. Scott Napper and ER2523 provided by NEB in the pMal protein fusion and purification system (NEB, Ipswich, Massachusetts, USA). All *E. coli* cells were grown in either liquid or solid Luria-Bertani (LB) medium (BD Difco Laboratories, Franklin Lakes, New Jersey) with 50 μg/mL or 100 μg/mL Ampicillin respectively (Sigma-Aldrich, St. Louis, Missouri, USA). Both solid and liquid cultures of *E. coli* were incubated at 37°C with liquid cultures being agitated at 200 rpm. *H. somni* AVI1 [35] provided by the laboratory collection was grown in either liquid Brain-Heart Infusion (BHI) (BD Difco Laboratories) or Tryptone Soya Agar (TSA) with 5% sheep blood agar (Oxoid Inc. Nepean, Ontario, Canada). Plates were incubated at 37 °C with 5 % CO_2 while liquid cultures were grown at 37 °C in plastic flasks (Corning Inc., Corning, New York, USA) with agitation at 200 rpm.

The plasmids used (Table 4-1) were pGH433His provided by the laboratory collection and altered by Dr. C. A. Madampage for the addition of the Histidine tag (His^6-tag) and pMAL-c5X purchased from NEB (NEB E8200S) for the purpose of Maltose-binding protein (MBP) fusion. A Leukotoxin (LktA) fusion plasmid pAA352, provided by the laboratory collection, was used for cloning of the gene coding for Tbp2.

Table 4-1 Plasmids used in this study

Plasmid	Genotype and Phenotype	Source
pGH433His	His tag/amp^R	Dr. A. Potter (VIDO-Intervac) [35]
pAA352	Lkta fusion/amp^R	Dr. A. Potter (VIDO-Intervac) [71]
pMAL-c5X	MBP fusion/amp^R	NEB

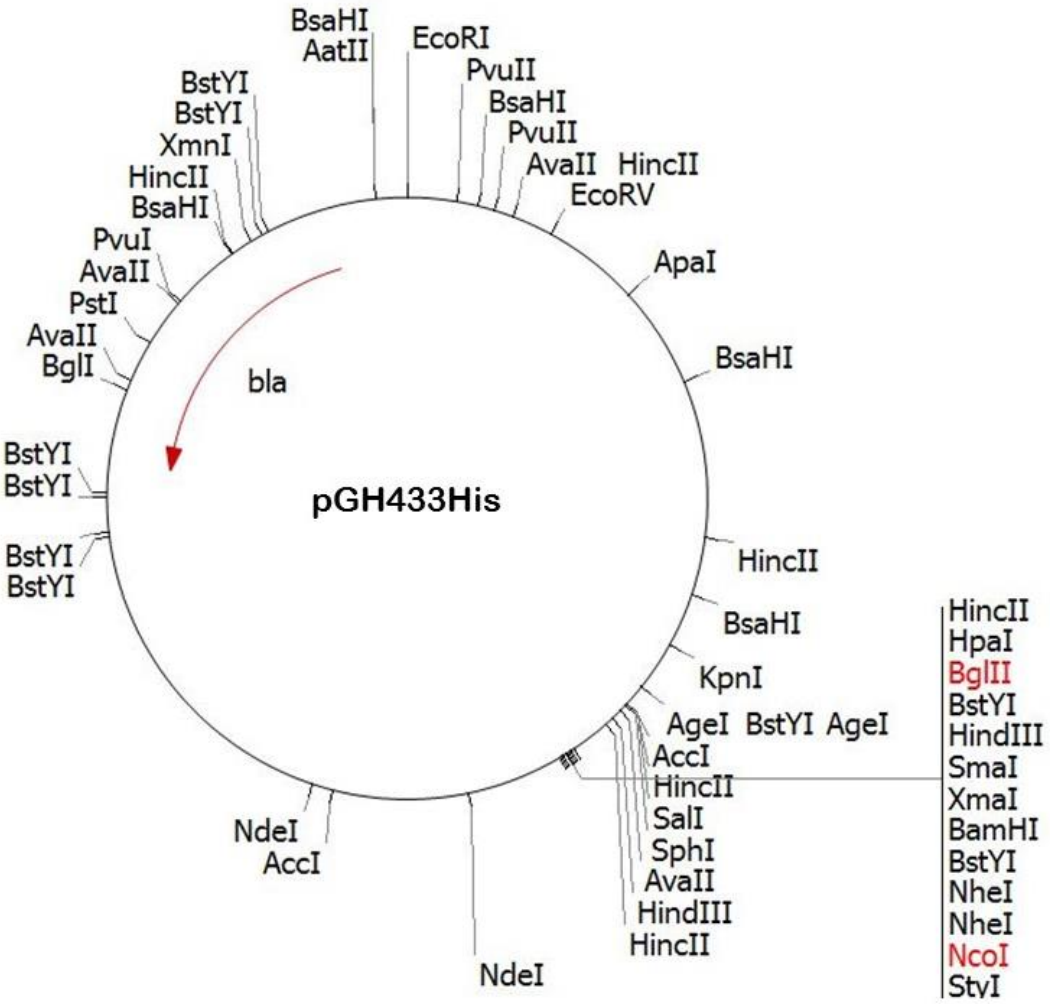


Figure 4-1 Plasmid map of pGH433His. Restriction sites used, BglII and NcoI, are indicated in red. *bla*: ampicillin resistance gene.

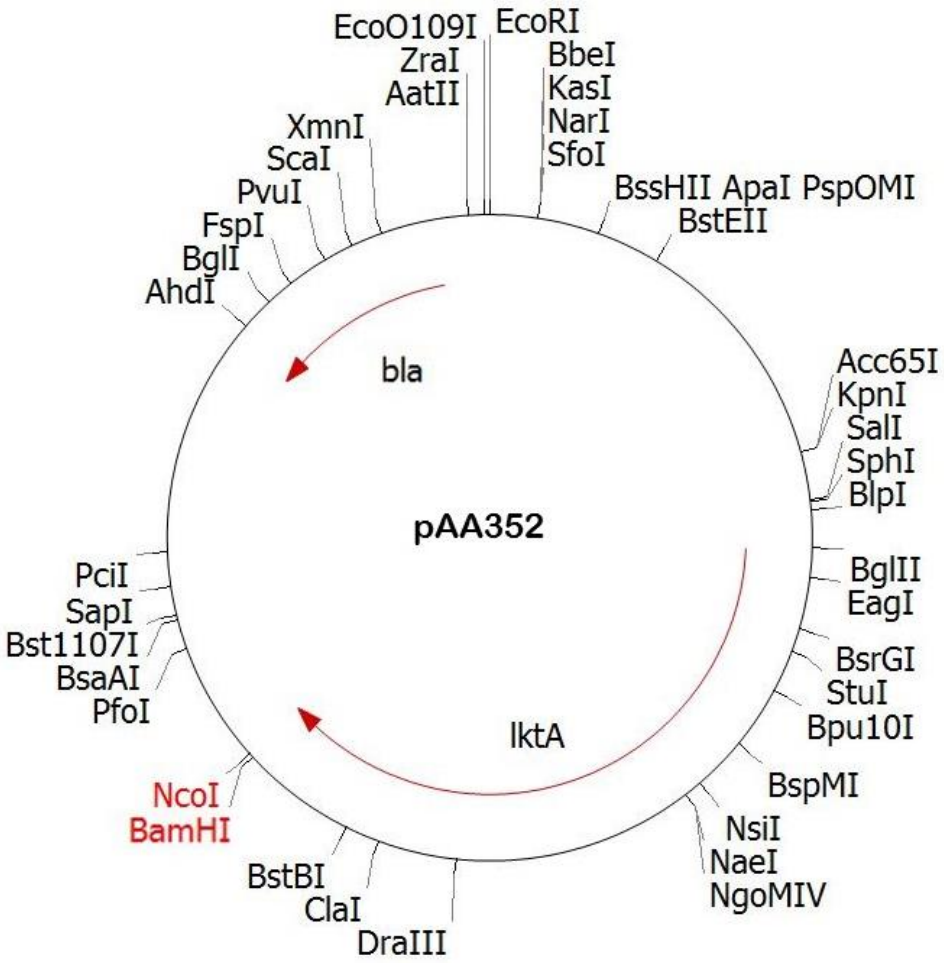


Figure 4-2 Plasmid map of pAA352. Restriction sites used, NcoI and BamHI are indicated in red. *bla*: ampicillin resistance gene; lktA: leukotoxin fusion gene.

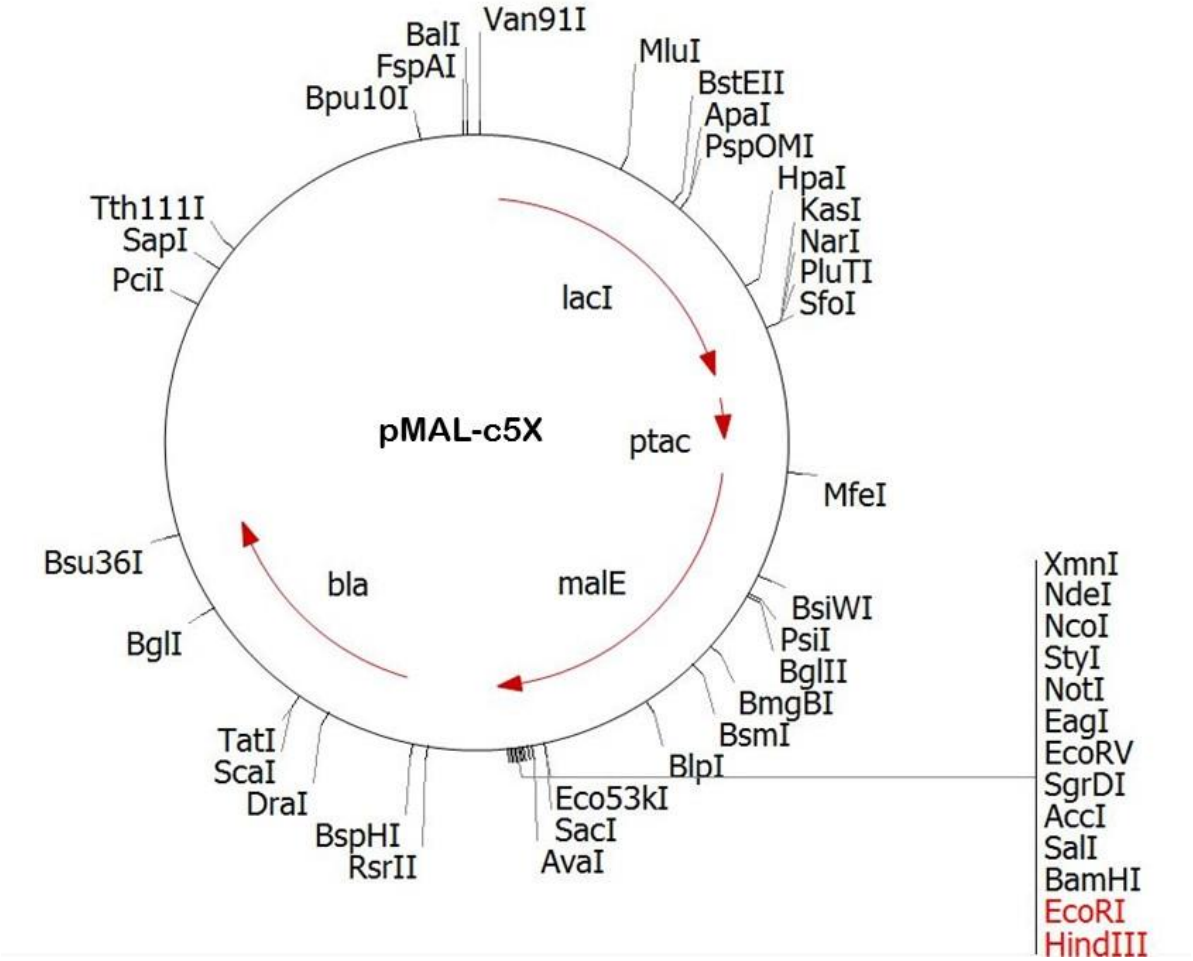


Figure 4-3 Plasmid map of pMAL-c5X. Restriction sites used, EcoRI and HindIII, are indicated in red. *bla*: ampicillin resistance gene; *malE*: gene encoding the maltose-binding protein (MBP); *lacI*: gene coding for the Lac repressor; ptac: promoter.

4.2 Cloning and expression

Cloning and expression of the genes coding for Receptor 1372, Tbp1 and Tbp2 was modified from Madampage et al. (2015). Polymerase chain reaction (PCR) amplification of all three genes used genomic DNA from the strain AVI1 using the primers described in Table 4-2. PCR was done using a PTC-100 Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) and consisted of an initial denaturation at 98 °C for 5 min, then 30 cycles of denaturation at 98 °C for 10 s, annealing at 63 °C for 30 s, and extension at 72 °C for 2 min. A final extension was carried out at 72 °C for 5 min. The annealing temperature and extension time varied slightly for each gene depending on the primers (refer to Table 4-2). Once PCR was complete, fragments were separated on electrophoresis in a 1% agarose (Sigma-Aldrich) gel. Receptor 1372 insert DNA and pGH433His vector DNA were then double digested with appropriate enzymes (NEB) (Table 4-2). The gene coding for Receptor 1372 was cloned using the pGH433His vector. Cloning of *tbp1* was attempted using the pGH433His, pAA352 and pMAL-c5X vectors (Table 4-2). Attempts at cloning *tbp2* used the pAA352 and pMAL-c5X vectors (Table 4-2). Digests were incubated at 37 °C for 1 hour or for 15 minutes when using high-fidelity enzymes. Electrophoresis separated the fragments in a 1% agarose gel then the DNA fragments were recovered via gel purification (Qiagen). In order to estimate the relative amount of DNA recovered, 4 µL of digested insert and vector DNA was separated by electrophoresis on 1% agarose. Once a concentration was estimated, ligation of the insert and vector DNA was done by incubation of digested DNA with 400 units of T4 DNA Ligase (NEB) overnight at 16 °C. Ligation mixes were then mixed with competent DH5αF'IQ (M15, ER2523 and BL21Plys for Tbp1 and Tbp2, refer to Table 4-2) *E. coli* cells while including a control of cells without DNA. Mixes were incubated on ice so the DNA adhered to the cell surface and heat shocked for 90 seconds at 42 °C during which the cells took up the DNA.

The cells cooled on ice for 2 minutes and were incubated in LB for 1 hour at 37 °C. Cells were harvested by centrifugation for 10 minutes at 200 rpm after which the cell pellet was resuspended in about 200 µL of leftover supernatant before being spread on LB-ampicillin agar to select for cells containing the plasmid. Viability of competent cells was checked by plating on LB agar and all plates were incubated overnight at 37 °C.

For all three genes, colonies positively selected from the ampicillin plates were screened by colony PCR and plasmid miniprep. The first screening method used was colony PCR where 16 selected colonies were streaked on new LB-ampicillin plates. The plates were incubated overnight at 37 °C and samples of each streak were immersed in 50 µL of H_2O (Sigma-Aldrich), boiled for 10 minutes at 95 °C and used in PCR with original primers to screen for colonies containing the insert. The second method of screening used in this study was the alkaline lysis method of DNA extraction [72] using the plasmid miniprep kit (Sigma-Aldrich). Six 5 mL aliquots of each overnight culture were harvested by centrifugation for 15 minutes at 3,400 rpm after which the pellet was resuspended and lysed according to the kit directions. Supernatant was loaded onto columns and centrifuged 1 minute at 12,000 x g. Each column was washed with 750 µL of the wash solution provided in the kit, centrifuged again and further centrifuged for 2 minutes at max speed. DNA was eluted from the column by the addition of 100 µL H_2O (Sigma-Aldrich) directly onto the membrane and centrifugation for 1 minute at 12,000 x g. Like elutions were pooled, concentrated and stored at -20 °C.

The screening of transformants containing *tbp1* and *tbp2* required the use of an adapted colony blot method [73] to screen the transformed colonies. While the transformation plates incubated, nitrocellulose disks (Bio-Rad) were prepared by soaking in Phosphate Buffered Saline

A (PBSA) (137 mM Sodium chloride, 2.7 mM Potassium chloride, 8.1 mM Sodium phosphate diabasic anhydrous, 1.5 mM potassium phosphate monobasic) + 0.1% Sodium dodecyl sulfate (SDS) (Bio Basic, Markham, Ontario, Canada). Once soaked the disks were incubated in a 65 °C water bath for 30 minutes to strip the membrane and make it permeable to IPTG. The disks were washed with ddH_2O and soaked in PBSA + 10 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) at room temperature for 30 minutes. The disks were dried overnight at 37 °C and the next day, they were gently placed on the transformation plates. Without shifting the disks, they were immediately lifted off and placed on fresh LB-ampicillin agar with the colony side up. Original plates and disks were incubated at 37 °C for 2 hours after which the disks were incubated with chloroform (Sigma-Aldrich) vapours for 15 minutes at room temperature. The cells were lysed with 10 mL Lysis buffer (0.1 M Tris pH 7.5, 0.17 M NaCl, 5 mM $MgCl_2$ $6H_2O$, 1.5% BSA Fraction V, 1 μg/mL DNase, 40 μg/mL lysozyme) and left overnight at room temperature with gentle shaking. The next day, disks were washed twice for 20 minutes in 1X Tris-buffered Saline (TBS) (1 M Tris base, 170 mM NaCl, pH 7.5) with gentle shaking. Once lysed, disks were wiped to remove cell debris and washed again with TBS. Disks were blocked for 30 minutes in 1% fish gelatin (Sigma-Aldrich), incubated with primary anti-MBP monoclonal antibody (NEB) diluted 1:4 for 1 hour and washed twice in Tris-buffered Saline + 0.1% Tween 20 (TBST) with shaking. The disks were incubated with a 1:2 dilution of secondary goat anti-bovine AP conjugated antibody (KPL, Gaithersburg, Maryland, USA) for 20 minutes and washed 3 times in TBST. Finally, they were developed in AP buffer (0.1 M Tris pH 9.6, 0.1 M NaCl, 50 mM $MgCl_2$ $6H_2O$) with Nitrotetrazolium Blue Chloride (NBT) (Sigma-Aldrich) and 5-Bromo-4-Chloro-3-indolyl phosphate p-toluidine salt (BCIP) (Sigma-Aldrich), ddH_2O was used to stop the reaction. Results were analyzed immediately after the reaction was stopped for best accuracy.

Transformants containing the *H. somni* gene of interest were further screened for protein expression through small-scale IPTG induction. An overnight culture of each clone was grown at 37 °C and diluted 1:10 the next day in LB-ampicillin. The diluted culture was incubated at 37 °C until it reached an optical density (OD) of 0.6 at 600 nm and then it was split into 2 tubes each containing 2.5 mL of culture where one served as the uninduced control. A final concentration of 1 mM IPTG was added to the induced sample before all samples were further incubated at 37 °C for 2 hours to allow the cells to be induced. After the 2-hour incubation, 1.5 mL aliquots were transferred into Eppendorf tubes and harvested by centrifugation for 5 minutes at 13,000 rpm. Cells were resuspended in 120 µL of 2x sample buffer (0.25 M Tris-HCl [Sigma-Aldrich] pH 6.8, 20% SDS, 20% Glycerol, 0.2% β-mercaptoethanol, Bromophenol Blue), boiled for 5 minutes at 95 °C and 5 µL was run on a 10% SDS-PAGE gel. Samples of induced and uninduced cells were also stored at -20 °C. Once it was determined they were expressing the correct-sized protein, samples were run on duplicate SDS-PAGE gels. One gel was transferred to nitrocellulose a membrane (Bio-Rad) for western blotting while the other was stained. After being transferred, the membrane was removed and washed in 1X TBS before being incubated for 20 minutes in 1X TBS + 1% fish gelatin then left at 4 °C overnight to block. The next day, the blocking solution was discarded and the appropriate antibody (anti-His monoclonal [Clontech, Moutain View, California, USA]/anti-MBP monoclonal [NEB]) was diluted 1:4 in 1X TBS + 1% fish gelatin and incubated for 1 hour at room temperature with gentle shaking. The membrane was washed with 1X TBS for 10 minutes and incubated with secondary goat anti-bovine antibody (KPL) diluted 1:2 for 1 hour at room temperature with gentle shaking. Once developed with the addition of AP buffer, BCIP and NBT, the membrane was washed with ddH_2O for 10 minutes and dried on the bench

overnight. Transformants that positively expressed Receptor 1372 and Tbp1 were grown overnight, diluted 1:1 in 40% glycerol and stored at -80 °C.

Table 4-2 Primers used in this study

Target Gene	Product Size (bps)	Vector	Annealing temp (°C)	Strain	Enzyme Sites	Sequence	*E. coli* strains used
TonB-dependent receptor (HSM_1372)	2388	pGH433His	63	2336	BglII NcoI	Forward: 5'CACCAGATCTGAAACAAGTAAAAATAAAGTTGAACGAC 3' Reverse: 5'CACCCATGGTTAGAATTCATACCCTAATTC 3'	DH5αF'IQ
Transferrin-binding protein 1 (HSM_0750)	2916	pGH433His	50, 58 and 60 (3.5-4 min extension)	2336	SmaI NcoI	Forward: 5'CGCGCGCCCGGGAAAAAAATGTCTACAAAACCTTTG 3' Reverse: 5'CGCGCGCCATGGTTAAAACTTCATTTCCATAC 3'	DH5αF'IQ M15
	3073	pAA352	50 (3.5 min extension)	AVI1	SmaI NcoI	Forward: 5'GGCGCGCCCGGGATCTACAAAACCTTTGTTTAAAC 3' Reverse: 5'GCTCCGCCATGG	ER2523 BL21pLys

						TTAAAACTTCATT TCCATACTCAC 3’	
	3063	pMAL-c5X	60 (4 min extension)	AVI1	EcoRI HindIII	Forward: 5’GCGCGCAATTCG AACTTAAGCCAAT AACATTG 3’ Reverse: 5’CGCGCGAAGCTT TTAAAACTTCATT TCCATACTCACGG 3’	ER2523 BL21pLys
Transferrin-binding protein 2 (HSM_0749)	1989	pAA352	50 (3.5 min extension)	2336	NcoI BamHI	Forward: 5’GGCGCGGGATC CGGCTTTTCGGTC TTGAGTG 3’ Reverse: 5’CGCCGGCCATGG TCATTTCTATTTCG ATAGTTCTTG 3’	M15
	1989	pMAL-c5X	60 (4 min extension)		EcoRI HindIII	Forward: 5’CGCGCGGAATTC CGGCTTTTCGGTC TTGAGTG 3’ Reverse: 5’ CGCCGGAAGCTTT CATTTCTATTTCG ATAGTTCTTG 3’	ER2523 BL21pLys

4.3 Protein purification

The TonB-dependent receptor 1372 was purified using a modified Ni-NTA column protein purification method [35]. *E. coli* DH5αF'IQ that positively expressed the receptor was used to inoculate 10 mL of fresh LB containing 100 μg/mL ampicillin, grown overnight at 37 °C and diluted 1:50 in warm 490 mL LB-ampicillin broth. Once the culture reached an OD_{600} of 0.6, 1 mL was collected as a non-induced sample. A final concentration of 1 mM IPTG was added to the remaining culture and both cultures were grown aerobically at 37 °C for 2-3 hours with shaking at 200 rpm. Cells were harvested by centrifugation at 6,500 rpm for 20 minutes at 4 °C, resuspended in 4 mL of 1X Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0) and stored overnight at -80 °C. The next day, the tube was thawed at room temperature. One mL of 10 mg/mL lysozyme was then added to the thawed pellet along with a sterile stir bar and the pellet stirred on ice for 30 minutes. The cells were lysed by sonication using Sonics Virba Cell (Betatek Inc. North York, Ontario) at 40%, 5 times for 30 s using a ½ inch probe. The lysed cells were centrifugated at 10,000 rpm for 30 minutes at 4 °C. The pellet was washed in 1X Buffer with the same centrifugation conditions before the final pellet was resuspended in 4 mL 2X Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, 8 M Urea, pH 8.0) and stirred on ice for 1 hour. The supernatant containing protein was obtained following centrifugation at 10,000 rpm for 30 minutes at 4 °C and stored at -20 °C. To purify the protein from the supernatant, 1 mL of Ni-NTA resin (Qiagen, Hilden, Germany) was added to the 4 mL lysate, left on a Nutator for 1 hour and gently loaded onto an empty column (BioRad). Flow-through was collected before the column was washed twice with 4 mL of Buffer C (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M Urea, pH 6.3) and eluted 4 times with 0.5 mL Buffer D (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M Urea, pH 5.9). Finally, a second elution was carried out using 0.5 mL Buffer E (100 mM NaH_2PO_4, 10 mM Tris

Cl, 8 M Urea, pH 4.5) applied 4 times. All elutions were collected in falcon tubes (VWR) from which 10 μL of sample was taken and mixed with 20 μL of 2x sample buffer before being run on a 10% SDS-PAGE gel. Densitometry was used to determine the protein concentration found in the elutions by comparing the values to those of known BSA standards using the program ImageLab. Flow-through fractions that still contained protein were passed through the column a second time.

Purification of Tbp1 was carried out following the protocol outlined in the pMAL Protein Fusion and Purification System (NEB). A glycerol stock of strain BL21pLys expressing Tbp1 was used to inoculate 10 mL of Rich Broth (10 g Tryptone, 5 g Yeast Extract, 5 g NaCl, 2 g Glucose) that was incubated overnight at 37 °C. The overnight culture was used to inoculate 1 L of Rich Broth and grown until an OD_{600} of 0.6 was reached when the culture was split and one half induced with 1 mM IPTG. The induced and non-induced cultures were incubated for 2 hours at 37 °C. Once induced, a second sample was taken and the cells were harvested by centrifugation at 4,000 x g for 20 minutes in 3 sterile centrifuge bottles. The pellets were resuspended in 15 mL of column buffer (20 mM Tris-HCL, 200 mM NaCl, 1 mM EDTA) and stored in a falcon tube at -80 °C. The next day the thawed sample was sonicated to lyse the cells. The protein-containing supernatant was obtained through centrifugation at 20,000 x g for 20 minutes and was subsequently diluted 1:2 with column buffer. The amylose resin (NEB) was prepared by pouring into columns and washing twice with 4 mL column buffer. The lysate was loaded carefully onto the columns and the flow-through collected. The columns were then washed 4 times with 9 mL of warm column buffer while collecting the first 4 mL as 1 mL fractions. The protein was eluted with 10 mL of 50 °C column buffer containing 10 mM maltose and 1 mL fractions were collected. Elutions were also attempted using 50 mM maltose. Because BL21pLyse was not the strain recommended by NEB, *E. coli* ER2523 that expressed Tbp1 was used in a second purification with the addition of

protease inhibitor in order to avoid any possible degradation of the fusion protein. The protein was eluted using column buffer containing 100 mM maltose followed by the addition of 2 M Urea. Elution was also attempted using 4 M Urea and Isopropanol (Sam Attah-poku, VIDO-Intervac).

As an alternative to the amylose column, anion exchange was used as a pilot experiment to determine the purification protocol for the MBP-Tbp1 fusion protein. *E. coli* ER2523 that expressed Tbp1 was used for fresh large-scale inductions as described above. The sonicated supernatant from the fresh culture was purified using anion exchange chromatography and the protein found in the flow-through fractions. To further process the flow-through, samples were pooled together, centrifuged at 13,000 rpm for 15 minutes and both the pellet and supernatant individually washed with 2 M Urea, 1 M Guanidine-HCl and 10 mL PBS. The samples were centrifuged again and dissolved in 8 M Guanidine-HCl/4 M Urea before being diluted with buffer (50 mM PBS, 4 M Urea, 10 mM Maltose, 0.5 mM DOC, 1 mM EDTA, pH 8.0). The samples were further purified by size-exclusion chromatography using Superdex 200 prep grade resin and Buffer (50 mM PBS, 1 M Guanidine-HCl, 4 M Urea, 10 mM Maltose, 0.25 mM DOC, 1 mM EDTA, pH 8.0). In continuation of locating the protein, the original pellet was explored further using an expanded version of the processing protocol. The sonicated pellet was resuspended in ddH_2O and large pieces of protein gently broken up with a pipette. The sample was centrifuged at 13,000 rpm for 15 minutes before buffer (50 mM PBS, 0.5 mM DOC, 10 mM Maltose, 1 mM EDTA, pH 7.5) was added to the pellet and mixed. The pellet was washed with the addition of 2.4 g solid Urea to a final concentration of 2 M, 10 mL of 50 mM PBS to dilute the DOC concentration and 2.87 mg of solid Guanidine-HCl to a final concentration of 1 M Guanidine-HCl and the solution mixed and heated for 1.5-2 hours. The pellet was separated from the supernatant by centrifugation at 13,000 rpm for 15 minutes and 15 mL of warm 8 M Guanidine-HCl/4 M Urea was added to dissolve the

insoluble material. The sample was heated, mixed for 1 hour and the supernatant was separated from the pellet by centrifugation at 13,000 rpm for 15 minutes. The supernatant was then diluted with buffer (50 mM PBS, 4 M Urea, 10 mM Maltose, 0.5 mM DOC, 1 mM EDTA, pH 8.0) to a final buffer concentration of 50 mM PBS, 1 M Guanidine-HCl, 4 M Urea, 10 mM Maltose, 0.5 mM DOC and 1 mM EDTA. The sample was concentrated using 15 mL x 50 kDa concentrators (Millipore). Fractions containing protein were pooled together and further purified by size-exclusion chromatography as above. The final fractions were pooled based on their relative purity on a 7.5% SDS-PAGE gel into 90% and 85% pools and concentrations of each were determined by densitometry (ImageLab) using BSA standards as described above.

Transformants expressing Tbp2 were not obtained when using either of the pAA352 or pMAL-c5X vectors. A previous contruct that contained the HS25 *tbp2* gene was used to purify Tbp2. The strain was streaked from a glycerol stock onto LB agar containing 100 μg/mL ampicillin and grown overnight at 37 °C. Ten mL of fresh LB-ampicillin was inoculated with fresh colonies. Once grown overnight, the 10 mL culture was added to 990 mL of LB-amp broth and grown until an OD_{600} of 0.6 was reached. A sample of uninduced culture was taken before the remaining culture was induced with 1 mM IPTG final concentration and incubated for 2-3 hours after which an induced sample was taken. To harvest the cells, the culture was centrifuged at 4,000 x g for 15 minutes and the pellet resuspended in 4 mL of 25% sucrose and 50 mM Tris (pH 8.0). The resuspended pellet was stored overnight at -80 °C. The next day, the pellet was thawed at room temperature before 10 mg/mL lysozyme was added and the sample placed on ice for 15 minutes. Fresh RIPA/TET (5:4 RIPA [20 mM Tris pH 8.0, 0.3 M NaCl, 2% Deoxycholic acid, 2% IGEPAL (NP-40)] TET [0.1 M Tris pH 8.0, 50 mM EDTA, Triton X-100]) was added at a volume of 30 mL to the lysed pellet. Once mixed and left on ice for 5 minutes, the protein was obtained by

sonication at 80% 5 times for 30 s. Once sonicated, the cells were harvested by centrifugation at 12,000 rpm for 20 minutes at 4 °C. The protein containing pellet was resuspended in 0.5 mL of 4M Urea and stored at 4 °C or run directly on a 10% SDS-PAGE gel. The concentration of the protein was determined with densitometry (Imagelab) by comparing the bands to known BSA standards.

4.4 Immunization and infection of cattle

Each 2 mL vaccine dose for cattle contained 250 μg of CpG2007, 30% Emulsigen (MVP Laboratories Lot# 2700), 100 μg of each protein and was made up to 2 mL with 0.1 M PBSA. Mice received a 40 μL dose containing 10 μg of CpG2007, 30% Emulsigen, 10 μg of each protein and 0.1 m PBSA. PBSA was the first reagent added followed by the proteins to ensure they remained in solution and did not precipitate. Following the protein, CpG2007 was added before finally adding the Emulsigen. The vials were sealed, thoroughly mixed, labelled and stored at 4 ^{0}C for use the next morning. Cattle vaccines were delivered sub-cutaneously while murine immunizations were delivered intra-muscularly.

A previous challenge experiment determined the optimal amount of *H. somni* AVI1 to be 10^8 colony-forming units (CFU)/mL. Calves were only moderately ill when challenged with 10^8 and in order to increase the stringency of infection, 7.5 x 10^8 CFU/mL of *Histophilus somni* AVI1 was used for the preliminary trial testing Receptor 1372. When testing Receptor 1372 along with Tbp1 and Tbp2, the challenge was 3.75 x 10^9 CFU/mL. All challenges were administered to the calves intravenously following blood sampling. These samples of blood were taken to obtain PBMCs and serum at the times of first immunization, boost, challenge, and post-mortem. All animals were examined daily and clinical scores, temperatures and weights recorded.

This study was randomized, controlled and blind. With the aid of a random numbers generator, calves were assigned to treatment groups. All calves were individually identified with an ear tag and housed in the same pen throughout the study. Investigators were kept unaware of the treatment status of the individual animals until the initiation of the data and statistical analysis.

4.5 PBMC isolation, spleen processing and cell stimulation

To determine if TonB-dependent proteins had the capacity to induce both cellular and humoral immune responses, a pilot cattle trial was done by immunizing calves with Receptor 1372 (Figure 4-4). Both the immunized and control groups contained 8 calves each and on day 0, they received the first immunization containing 100 μg of Receptor 1372. On day 30, the calves received a boost immunization and 2 weeks later on day 44, they received an intravenous challenge of *H. somni* AVI1 at about 10^9 CFU/mL. One to two weeks later, depending on the clinical symptoms, the animals were euthanized for post-mortem sampling. Peripheral blood mononuclear cells (PBMCs) were isolated from 100 mL blood samples. Peripheral blood mononuclear cells were isolated as described [74]. The buffy coat containing the PBMCs was obtained by centrifugation of blood samples at 2,500 rpm for 20 minutes without brake. This buffy coat was then mixed with 20 mL PBSA + 2.7 mM Ethylenediaminetetraacetic acid (EDTA) before being gently layered on 12 mL of Ficoll (GE Healthcare, Mississauga, Ontario, Canada), topped up to 50 mL with PBSA + EDTA and centrifuged at 3,000 rpm for 20 minutes without brake. The buffy coat was again mixed with 20 mL PBSA/ 2.7 mM EDTA and topped up to 50 mL. Washing PBMCs consisted of centrifugation at 1,200 rpm for 10 minutes, discarding the supernatant and gently resuspending cells by knocking the tubes together before filling them with 50 mL PBSA. This was repeated 3 times before cells were resupended in 5 mL of PBSA. Cells were counted by diluting 1:100 in PBSA and using a Sceptre cytometer (Milipore, Billerica, Massachusetts, USA)

with 40 μm sensors (Millipore) or a hemocytometer with a 1:2 dilution of cells in Turk's (Millipore) solution. PBMCs were used in proliferation assays as discussed in section 4.6.

A second cattle trial took place to characterize the immune responses and protective capacity when immunized with Receptor 1372, Tbp1 and Tbp2. The animal trial was designed similarly to the above mentioned cattle trial with animals receiving 100 μg each of Receptor 1372, Tbp1 and Tbp2. Blood was sampled on days 0, 30, 44 and 50, and PBMC isolation followed the same protocol as described above.

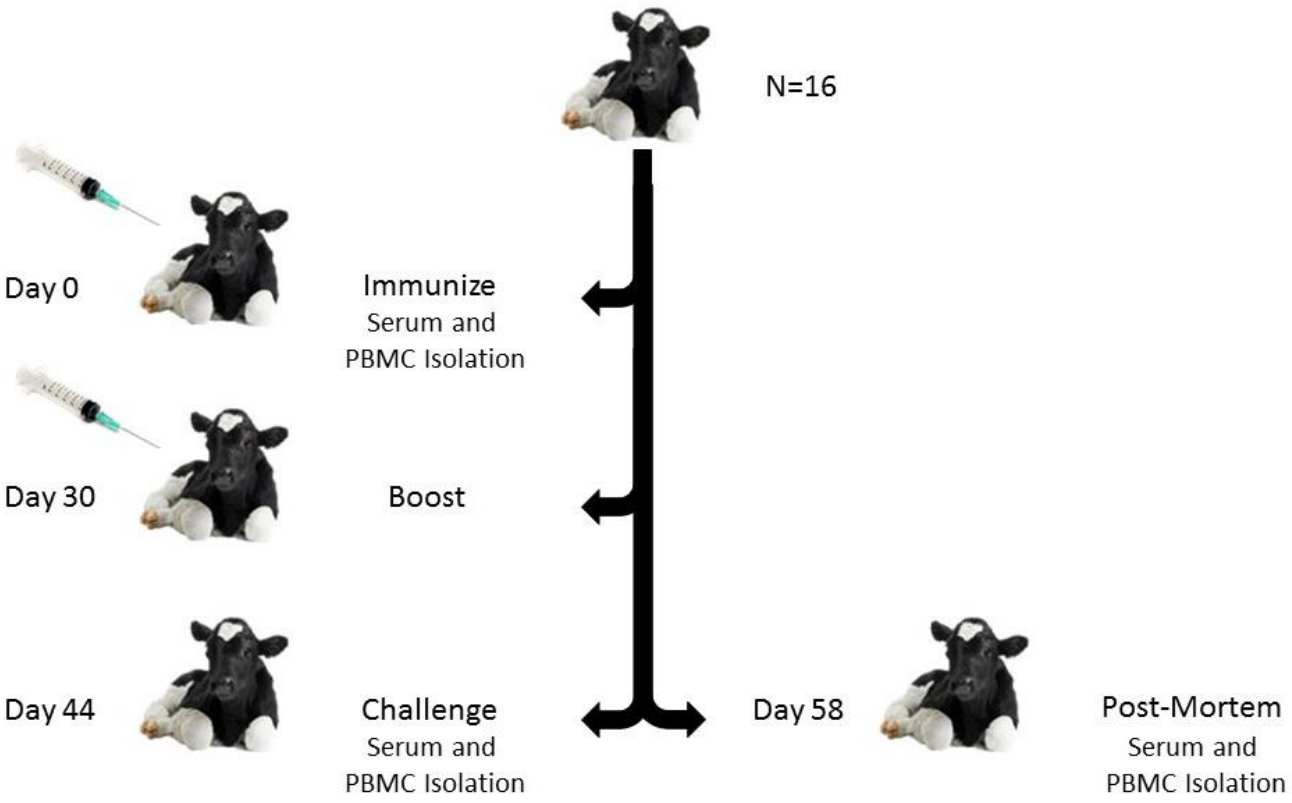


Figure 4-4 Cattle trial protocol. Cattle were split into 2 groups: vaccine and control, each consisting of 8 animals. Animals were immunized on Day 0 and day 30. The intra-venous challenge of *H. somni* AVI1 was given on Day 44. Post-mortem followed 1-2 weeks after challenge. Samples of blood were taken at each time point for PBMC isolation.

A murine trial determined if receptor 1372, Tbp1 and/or Tbp2 were capable of inducing a cellular response in mice (Figure 4-5). In this trial, all mice were randomly assigned to both group and cage. On day 0, 2 groups of five mice were immunized with 10 μg each of Receptor 1372, Tbp1 and Tbp2. On this same day one group of five control mice was sacrificed and their spleens removed for processing following an adapted spleen processing method from Benencia et al. [75]. On day 14, five of the immunized mice received a second immunization while the remaining five, along with five controls, were euthanized and spleens processed. On day 28, all remaining animals were euthanized and splenocytes isolated. Spleens were immediately placed in 15 mL Minimal Essential Media (MEM) (Sigma-Aldrich) + 10 mM HEPES (Lifetech, ThermoFisher Scientific, Waltham, Massachusetts, USA), 50 U/mL Penicillin/Streptomycin (Gibco, ThermoFisher) and kept on ice. To isolate splenocytes, a spleen was placed in a sterile petri dish with 5 mL MEM and a 40 μm strainer (ThermoFisher). Once moved to the strainer, the spleen was cut into pieces with sterile scissors and gently pushed through using a sterile glass plunger until there was little to no spleen left. The strainer was rinsed multiple times with the 5 mL of MEM before being moved to a 15 mL falcon tube. The petri dish was also washed to remove all cells. Cells were centrifuged at 311 x g for 10 minutes at 4 °C, media poured off or carefully removed with a pipette and the pellet resuspended by knocking tubes together and adding 5 mL of AIM V (Gibco, ThermoFisher) + 50 U/mL Penicillin/Streptomycin, 0.1 μM β-mercaptoethanol, 10% Fetal bovine serum (FBS) (Gibco, ThermoFisher). Cells were counted using the hemocytometer by diluting 1:4 in PBSA and 1:2 in Turk's solution. Isolated splenocytes were re-stimulated with each protein for RNA isolation (see section 4.7). Once counted, cells were diluted or concentrated to 1 x 10^6 cells/well and allowed to stabilize for 1 hour at 37 °C with 5% CO_2 while each protein was diluted to 1 ng and 10 ng. Concanavalin A (ConA) (Sigma-Aldrich) was diluted to 2 μg/mL. Once stabilized, 100 μL of cells,

in triplicate, were incubated at 37 °C with 5% CO_2 for 20 hours with 100 µL of each antigen. ConA was used as a positive control and media for a negative control.

This study was randomized, controlled and blind. With the aid of a random numbers generator and a computer spreadsheet (Microsoft Excel), mice were randomly assigned to both cage and treatment groups. Equal numbers of mice from each treatment group were housed in each cage and within each cage; mice were individually identified with randomly assigned ear notches. Investigators were kept unaware of the treatment status of the individually animals until the initiation of data and statistical analyses.

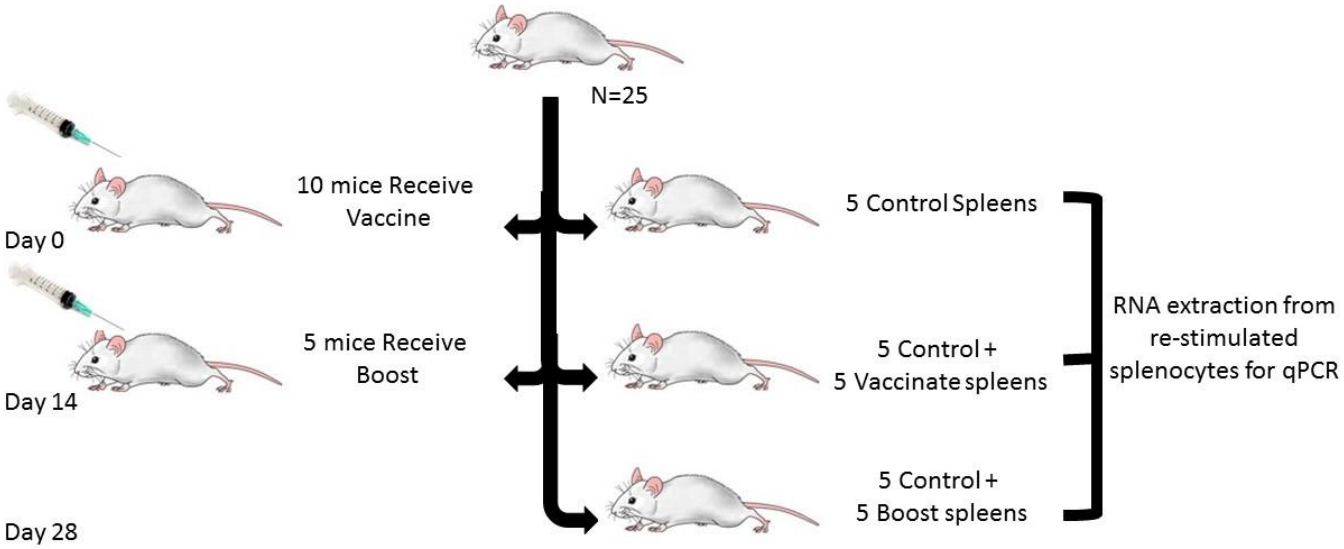


Figure 4-5 Murine trial protocol. Mice were split into 5 groups: 3 control groups, 1 vaccine group and 1 boost group. On day 0, a control group was sacrificed as a negative control, while the vaccine and boost groups were immunized. On day 14, the vaccine group and a second control group was sacrificed while the boost group received a second immunization. On day 28, the boost group and remaining control group were sacrificed. Spleens were removed from all sacrificed animals and processed to isolate the splenocytes for qPCR analysis.

4.6 CFSE staining and proliferation assays

For both cattle trials, isolated PBMCs were stained for proliferation assays following the CFSE staining method [76]. Carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) was spun down, reconstituted with 18 µL of Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) for a final concentration of 5 mM and centrifuged briefly again. PBMCs were diluted or concentrated to 2 x 10^7 cells/mL in PBSA and allowed to adjust to room temperature. CFSE was diluted to a working concentration of 5 µM, 1 mL added to each cell suspension, mixed rapidly and incubated in a 37 ^{0}C water bath for 10 minutes. The reaction was immediately stopped by the addition of 2 mL FBS and gentle inversion to mix thoroughly. Cells were incubated at room temperature in the dark for 15 minutes after which cells were harvested by centrifugation at 400 x g for 8 minutes. PBMCs were washed thoroughly twice by resuspension in 14 mL room temperature MEM complete (MEM + 0.05 mg/mL Gentamycin sulphate [Bio Basic], 10% FBS, 50 µM β-mercaptoethanol, 5 mL sodium pyruvate [Lifetech, ThermoFisher], 5 mL Non-essential amino acids [NEAA] [Lifetech, TermoFisher], 5 mL HEPES) and centrifuged at 400 x g for 8 minutes. Supernatants were discarded while cells were resuspended in 14 mL MEM complete and incubated at 37 °C plus 5% CO_2 for 30 minutes. Once incubated, PBMCs were harvested by centrifugation at 350 x g for 8 minutes, resupended in 5 mL MEM complete and diluted 1:10 in PBSA for counting with either the Scepter or hemocytometer as mentioned above.

PBMCs, stained with CFSE, were set up for proliferation assays using receptor 1372, Tbp1 and Tbp1 to stimulate the cells. Cells were diluted to a final amount of 1 x 10^6 cells/mL and 100 µL plated with each treatment. The treatments for the pilot experiment consisted of: 1 ng, 2 ng, 10 ng, 20 ng and 30 ng of receptor 1372, 2 µg/mL ConA and media to determine the ideal concentration of protein for re-stimulation. For the final cattle trial, the PBMCs were treated with

20 ng of each protein, 2 μg/mL ConA and media. Cells were incubated with the treatments at 37 ºC plus 5% CO_2 for 4 days. On day 4 of incubation, proliferation was measured by BD FACSCalibur (Bio-Rad Laboratories) and CellQuestPro. Analysis was done by using the programs Kaluza and Graphpad Prism with the gating strategy shown in Figure 4-6.

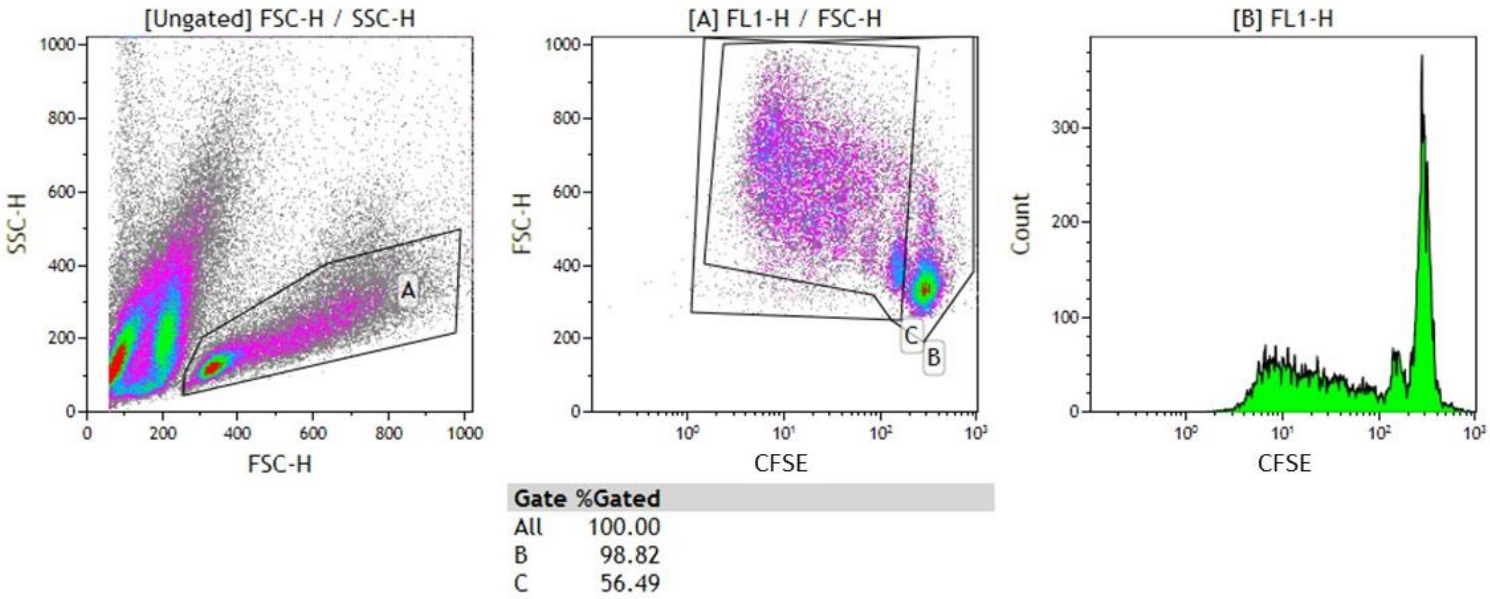


Figure 4-6 PBMC gating strategy. Proliferation of PBMCs was measured using flow-cytometry. The gating strategy consisted of gating the PBMCs using gate A, where cells were measured by the side-scatter (SSC-H) and forward-scatter (FSC-H). This was followed by gating of the proliferating cells (gate C) from the total population (gate B). Here, cells were graphed using forward-scatter and the amount of CFSE fluorescence. The histogram shows the number of proliferating PBMCs (Count) by quantity of intracellular CFSE.

4.7 Gene expression analysis by qPCR

Analysis of gene expression through Quantitative PCR (qPCR) began with isolation of RNA from re-stilumated murine splenocytes using an RNeasy isolation kit (Qiagen). Stimulated splenocytes were pooled into Eppendorf tubes and harvested by centrifugation at 400 x g for 15 minutes at 4 °C before being lysed with 400 µL of lysis buffer (RLT). The samples were then vortexed for 1 minute each and frozen at -80 °C until isolation. For isolation, the lysed cells were thawed on ice and 1 volume of 70% ethanol added, mixed and 700 µL transferred to a column placed in a 2 mL collection tube. Columns were centrifuged at 8,000 x g for 30 s after which the flow-through was discarded and 700 µL of Buffer RW1 added before centrifugation at 8,000 x g for another 30 s. The flow-through was discarded and column washed with Buffer RPE and centrifuged again. Another 500 µL of RPE was added to the column, which was centrifuged at 8,000 x g for 2 min. At this point, it was imperative to keep the workspace and pipettes RNase-free. The column was placed into a new 1.5 mL collection tube, 30 µL of RNase-free water (Sigma-Aldrich) was added and the column was left to sit for 1-2 minutes before centrifugation at 8,000 x g for 1 min to elute the RNA. The elution step was repeated using the 30 µL of eluate from the previous step. Concentration and purity of the RNA was determined using the Nanodrop ND-1000 Spectrophotometer (ThermoFisher) before being reverse transcribed using the High-Capacity Reverse Transcription kit (Applied Biosciences, Foster City, California, USA). Each RNA sample was diluted to 500 ng with the RNase-free water (Sigma-Aldrich) while also including plate controls. A mastermix was made with 10X RT Buffer, 25X dNTO mix (100 nm), 10X RT random primers and MultiScribe reverse transcriptase according to the kit manual. Mastermix was added to the samples at a volume of 5.8 µL. The reactions were run on a PTC-100 Thermal Cycler (Bio-

Rad) with the following steps: step 1 at 25 °C for 10 min, step 2 at 37 °C for 120 min and step 3 at 85 °C for 5 min. All cDNA was stored at -20 °C until set-up for qPCR reactions.

Gene expression was measured from the splenocyte cDNA using the qPCR method [77]. The cDNA was diluted to 100 µL with RNase-free water. A mastermix was created using Kapa Sybr Fast qPCR Mastermix ABI Prism (Kapa Biosystems, Wilmington, Massachusetts, USA), forward and reverse primers specific for Interleukin-4 (IL-4), Interferon-gamma (IFNγ) or Ribosomal protein L19 (RPL19) (Table 4-6) and RNase-free water. Two µL of cDNA was added to qPCR plates (Applied Biosystems) and 13 µL of the above mastermix. Plate covers (Applied Biosystems) sealed the plates, which were centrifuged briefly and stored overnight at -20 °C. The next day, plates were thawed at room temperature in the dark, centrifuged briefly and checked for air bubbles before being set up on StepOnePlus Real-Time PCR Systems (Applied Biosystems). Once set-up each plate was measured for the expression of IL-4 or IFNγ. Analysis was carried out using Microsoft Excel and Graphpad Prism.

Table 4-3 qPCR primers

Gene target	Primer name	Tm (°C)	Sequence
IL-4	IL4 Forward	60.17	GAGAGATCATCGGCATTTTGA
	IL4 Reverse	59.88	GGTGTTCTTCGTTGCTGTGA
IFNγ	IFNg Forward	60.3	CACGGCACAGTCATTGAAAG
	IFNg Reverse	59.3	TCATGTCACCATCCTTTTGC
RPL19 (control)	RPL19 Forward	59.31	CCTCATGCCCACAAGGTACT
	RPL19 Reverse	59.99	CCCTGTCTTGTATCGCTCCT

4.8 ELISA assays

Serum titres for all cattle trials were measured with enzyme-linked immunosorbent assays (ELISAs) adapted from the ELISA method described by Darshud et al [78]. Immulon 2 96U plates (ThermoFisher) were coated with 10 ng of each receptor 1372, Tbp1 and Tbp2 per well using Coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate) before being left overnight at 4 ^{0}C. The next day the plates were washed 6 times with tap reverse osmosis (RO) water, wells blocked with the addition of 100 µL 1X TBST + 0.5% fish gelatin (Sigma-Aldrich) and left at room temperature for 1 hour. While blocking the plates, cattle serum and control serum was diluted 1/40 and serially diluted 2-fold down a 96 well plate using 1X TBS. Once plates were blocked, they were washed again with tap RO water, the diluted serum moved to the blocked plate and left at room temperature for 2 hours. Plates were washed again before goat anti-bovine alkaline phosphatase (AP) conjugated secondary antibody diluted 1/2000 in 1X TBST was added to each well before being left at room temperature for 2 hours. Once plates were washed to remove secondary antibody, a 0.1 g/mL stock of P-Nitrophenyl Phosphate (PNPP) was diluted to 1 mg/mL in Substrate buffer (0.5 mM $MgCl_2$, 10 mL Diethanolamine) and added to the plates that were then left to develop in the dark at room temperature for up to 1 hour. Plates were checked every 10 minutes to ensure plates did not over-develop. Optical densities were measured using an iMark Microplate Reader (Bio-Rad) set to 405 nm. Serum titres were determined using Microsoft Excel through determination of the cutoff (average of negatives + 2 standard deviations). Using this cutoff value enabled the determination of titres based on the forecast formula.

The expression of IL-4 and IFNγ was confirmed with the use of cytokine ELISAs following the Duo-set ELISA Development System kit protocol (R & D Systems, Minneapolis, Minnesota, USA). In the event that IL-4 assays did not work, Interleukin-5 (IL5) was also used to confirm the

humoral response seen through qPCR. The capture antibody was diluted to 4.0 μg/mL in PBS and used to coat Immulon 2 96U plates overnight at room temperature. The next day, plates were washed 3 times with washing buffer (0.05% Tween 20 in PBS, pH 7.2 - 7.4) and blocked with appropriate blocking buffer (specific for each cytokine: 0.1-1% BSA in PBS) at room temperature for 1 hour. The plates were washed again and supernatant from stimulated murine splenocytes diluted 1:10 in Reagent diluent (specific for each cytokine: 0.1-1% BSA, 0.05% Tween 20 in TBS, pH 7.2 - 7.4) along with Standard (diluted to 2 ng/mL), in duplicate, and incubated at room temperature for 2 hours. Once plates were washed, detection antibody specific for the cytokine was diluted to 800 ng/mL in reagent diluent, added to the plates and left at room temperature for another 2 hours. After washing again, Strepdavidin-HRP (R & D Systems) was diluted in reagent diluent to working concentration on the bottle. These were then added to each well and left to develop at room temperature for 20 minutes in the dark. Again, the plates were washed and 100 μL of SureBlue TMB microwell peroxidase substrate (KPL) was added directly to each well and plates left for another 20 minutes in the dark. After development, 50 μL of Stop Solution (2 N H_2SO_4) was added to each well before the plates were read with a SpectraMax Plus microplate reader (Molecular Devices, Sunnyville, California, USA) set to 450 nm. Analysis was done using Microsoft Excel and Graphpad Prism.

4.9 Statistical analyses

All statistical analysis of serum ELISA used non-parametric t-test (Mann-Whitney) and qPCR results used two-way ANOVA with Sidak's multiple comparison test. Statistical analysis of cytokine ELISA used unpaired t-test t compare non-immunized and immunized groups. Statistics for proliferation assays used parametric t-tests in order to compare the immunized and non-immunized groups. All statistical analyses were completed using Graphpad Prism software.

Significant results were determined by P value where a P value greater than 0.05 is not significant and less than or equal to 0.05 is significant.

5.0 RESULTS

5.1 Gene cloning and purification of Receptor 1372

In order to obtain purified Receptor 1372 for use in animal trials, the gene coding for the receptor was amplified using PCR and the vector pGH433His was used for ligation. Following ligation, *E. coli* DH5αF'IQ were transformed. Transformants were screened using the colony PCR method previously described followed by further screening to identify clones expressing the protein. Once transformants were expressing the Receptor 1372, lysed cells were passed through a Ni-NTA column that binds the His^6-tagged protein. The use of SDS-PAGE identified elutions containing Receptor 1372 and the use of densitometry determined the concentrations.

Receptor 1372 was amplified from genomic *H. somni* AVI1 DNA, ligated, transformed and expressed. Once expressed, the protein was purified. Amplification of the receptor 1372 using the primers specified for pGH433His resulted in DNA bands of 2388 base pairs (bp) (Figure 5-1A). The ligated insert DNA was transformed into *E. coli* DH5αF'IQ and the transformants screened for the expression of Receptor 1372 using small-scale IPTG induction (Figure 5-1B). The protein was expressed at a size of 90 kD, as expected, by 2 transformants (1372_#4 and 1372_#9). Western blotting using an antibody specific for the His^6-tag of the protein confirmed the expression of Receptor 1372 (Figure 5-1C). Confirmed transformants were used to inoculate large-scale cultures in order to produce large amounts of protein. The protein was purified by passage through a Ni-NTA column that resulted in the protein being eluted in fractions 1-5 (Figure 5-2). Often the protein was also found in the flow-through due to the resin becoming saturated so it was passed through the column a second time. Thus, the gene coding for Receptor 1372 was cloned using the in-house vector pGH433His, expressed in *E. coli* DH5αF'IQ and the protein purified using Ni-NTA columns.

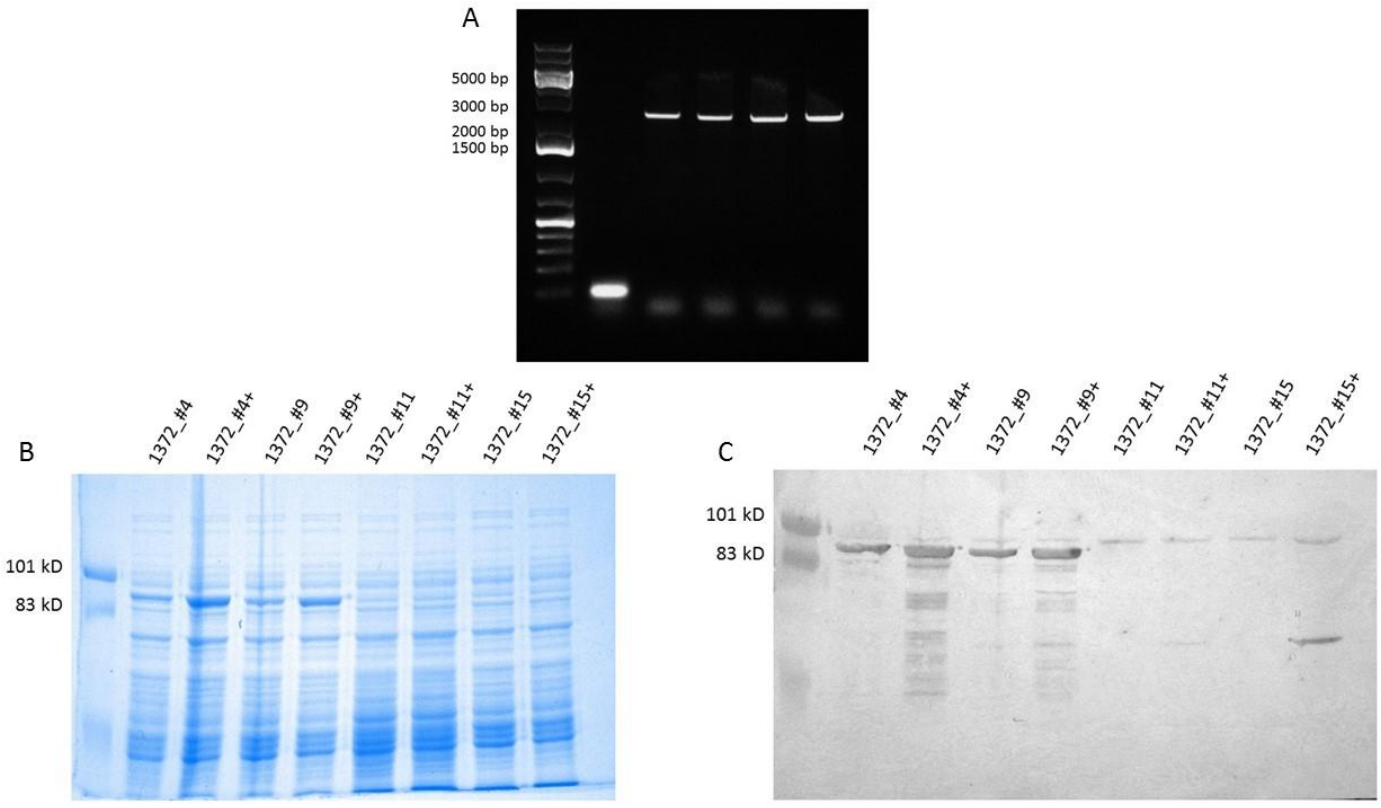


Figure 5-1 PCR and small-scale IPTG induction of Receptor 1372. (A) Amplification of receptor 1372 from the *H. somni* strain AVI1 using primers designed for ligation with vector pGH433His. (B) SDS-PAGE gel of small-scale induction where 2 clones (1372_#4 and 1372_#9) are expressing Receptor 1372 when induced with IPTG (addition of IPTG indicated by '+'). (C) Small-scale induction samples were transferred to nitrocellulose for western blot using anti-His[6] monoclonal antibody to confirm Receptor 1372 protein expression.

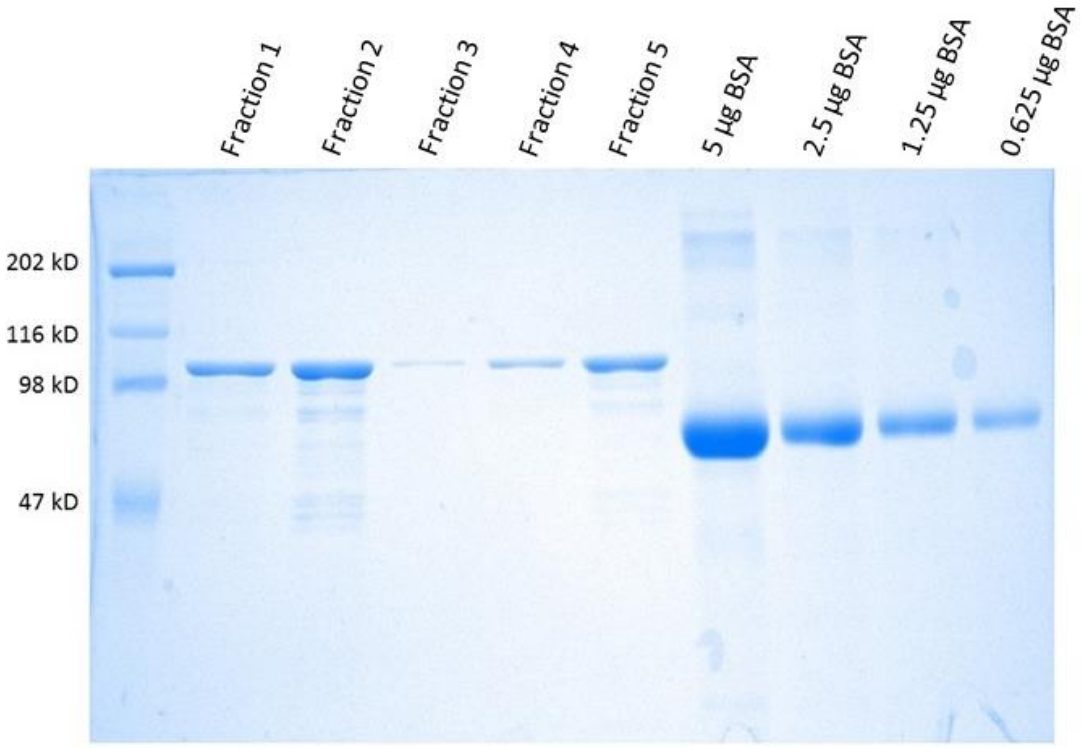


Figure 5-2 SDS-PAGE gel of purified Receptor 1372. Fractions 1 through 5 containing purified Receptor 1372. Densitometry used BSA standards to determine the concentration of protein in each elution. Elution 1 (0.3 µg/µL), Elution 2 (0.4 µg/µL), Elution 3 (0.04 µg/µL), Elution 4 (0.12 µg/µL), Elution 5 (0.36 µg/µL).

5.2 Gene cloning and purification of Transferrin-binding protein 1 (Tbp1)

In order to acquire purified Tbp1 for use in animal trials, *tbp1* was amplified and the DNA used for ligation with vectors pGH433His and pAA352. When these were unsuccessful, newly amplified DNA was ligated into the vector pMAL-c5X. Once ligated into pMAL-c5X, *E. coli* ER2523 and BL21pLys cells were transformed and then screened by colony blotting. Further screening identified transformants containing the gene of interest as expressing Tbp1. Once expression of Tbp1 was obtained, the protein was purified through various attempts including amylose columns that were unsuccessful. Affinity chromatography led to the development of a processing protocol that identified Tbp1 in flow-through and pellet samples. These samples were further purified by size-exclusion chromatography in order to obtain pools of purified Tbp1.

The cloning and purification of Tbp1 was important to obtain two pools of known concentration, 90% and 80%, used in the immunization of cattle and mice. The gene coding for Tbp1 was amplified using primers designed specifically for cloning into the vectors pGH433His and pAA352 (results not shown). These PCR fragments were then used for ligation and transformation into *E. coli* DH5αF'IQ and M15. However, the transformants that were isolated did not contain the insert gene so *tbp1* was amplified using primers specific for pMAL-c5X that resulted in DNA bands of about 3000 bp (Figure 5-3A). This DNA was used for ligation in pMAL-c5X and transformation into *E. coli* ER2523 and BL21pLys. Transformants were screened for Tbp1 expression immediately by colony blotting due to the large number of clones. This resulted in the identification of two transformants shown in Figure 5-3B. These two transformants were further screened by small-scale IPTG induction and both showed expression of the MBP-Tbp1 fusion protein at the expected size of 160 kD (Figure 5-3C). Western blot using anti-MBP antibody

that bound the MBP portion of the fusion protein confirmed protein expression (Figure 5-3D). Both transformants, along with 4 others had positive expression of Tbp1.

In order to produce larger quantities of the fusion protein, strain BL21pLys_Tbp1_1 was grown in 3 litres and the protein harvested as described for purification on amylose resin. The MBP binds strongly to the amylose resin but has a stronger affinity for the maltose used to elute the protein. This, however, did not appear to be the case and it was found that MBP-Tbp1 would bind to the resin and not elute regardless of the concentration of maltose used. Since the strain BL21pLys was not the strain suggested by NEB, the clone ER2513_Tbp1_2 was used for a second attempt. Protease inhibitor was added to this induction to avoid any degradation of the protein while the column was eluted with 100 mM maltose. Unfortunately, this, as well as elution with 4 M Urea and isopropanol did not yield results. A new culture of ER2523_Tbp1_2 was induced and harvested as described above to obtain the supernatant that was explored first through affinity chromatography. This led to the discovery that the fusion protein was in the flow-through (Figure 5-4A) while all the contaminants were binding to the column. Through some explorations of the sonicated pellet it was discovered that the fusion protein was there as well (Figure 5-4A). Once the processing protocol was developed through the analysis of supernatant and flow-through samples, it was applied to the flow-through fractions that were then further purified by size-exclusion chromatography (Figure 5-4B). From here, the sonicated pellet was processed in the same way and the results showed that the pellet contained much more fusion protein than the supernatant (Figure 5-4C). Using the processed pellet and flow-through samples, two pools of MBP-Tbp1 were made: 90% and 80% (Figure 5-4D). Thus, the gene coding for Tbp1 was cloned using the maltose fusion vector pMAL-c5X, expressed in *E. coli* ER2523 and purified using both anion exchange and size-exclusion chromatography.

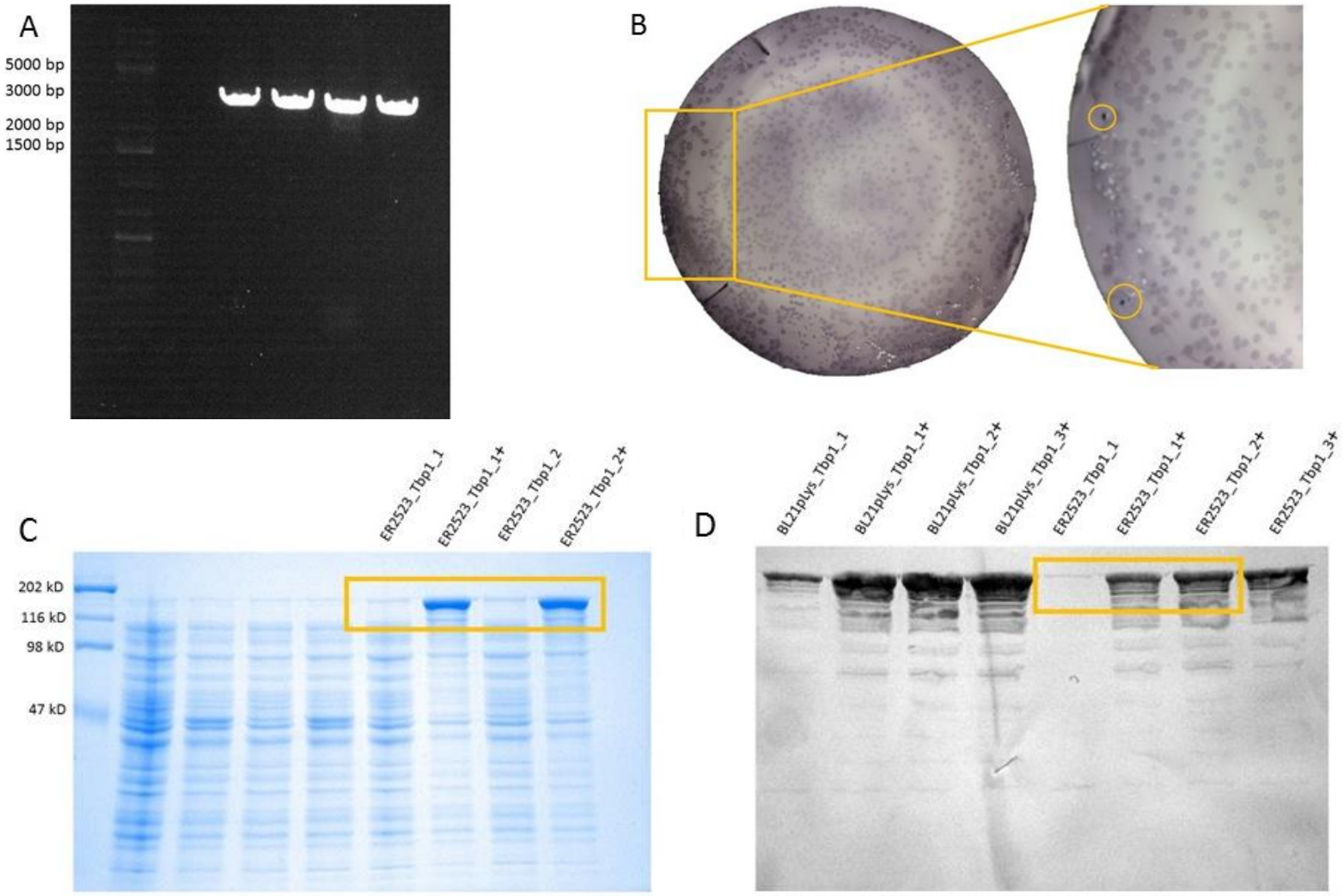


Figure 5-3 PCR, colony blot and small-scale IPTG induction of Tbp1. Amplification of Tbp1 from the *H. somni* strain AVI1 using primers designed for ligation with vector pMAL-c5X. (B) Colony blot after transformation with pMAL-c5X/Tbp1 ligation. Colonies expressing the MBP-Tbp1 fusion protein are indicated. (C) Small-scale IPTG induction of clones ER2523_Tbp1_1 and ER2523_Tbp1_2 where each shows expression of Tbp1. (D) Induced samples of ER2523_Tbp1_1 and ER2523_Tbp1_2 transferred to nitrocellulose for western blot to confirm protein expression with anti-MBP monoclonal antibody. IPTG induction is indicated by '+'.

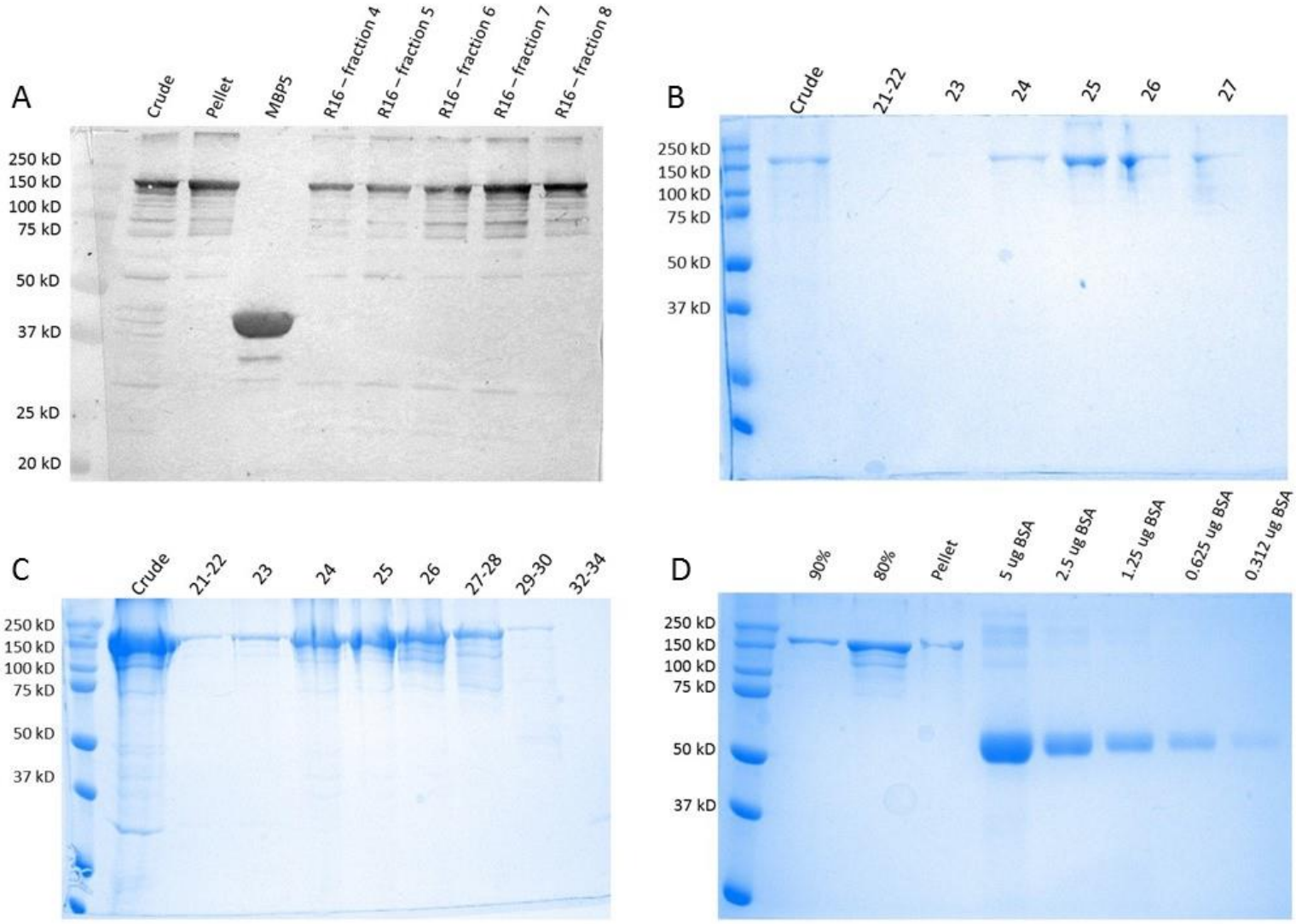


Figure 5-4 SDS-PAGE gels of purified Tbp1. (A) Western blot using anti-MBP monoclonal antibody showing crude processed pellet, original sonicated pellet, a MBP control, and flow-through fractions 4-8. The MBP-Tbp1 fusion protein was found in sonicated sample, sonicated pellet as well as in the flow-through from anion-exchange of sonicated supernatant. (B) SDS-PAGE gel of crude pellet and fractions 21-27 showing processed sonicated supernatant with some presence of the fusion protein. (C) SDS-PAGE gel of crude pellet and fractions 21-34 showing processed sonicated pellet with increased presence of purified MBP-Tbp1. (D) SDS-PAGE gel of purified fusion protein from processed sonicated pellet after size-exclusion chromatography. Fractions were pooled based on relative purity on SDS-PAGE into 90% and 80%. Processed pellet was also found to be quite pure. Densitometry used BSA standards to determine the concentration of protein in each aggregate preparation. Nintey % (0.7 μg/μL), 80% (2.8 μg/μL)

5.3 Gene cloning and purification of Transferrin-binding protein 2 (Tbp2)

In order to obtain purified Tbp2, many attempts at cloning were made including the amplification and subsequent ligation of *tbp2* into vector pAA352. Since this ligation resulted in *E. coli* M15 transformants expressing a smaller protein, the gene was ligated into pMAL-c5X. When the gene of interest was not found in these transformants, a decades-old construct, HS25_Tbp2, was used for the purification of Tbp2.

The cloning and purification of Tbp2 began with the use of a leukotoxin fusion vector, PAA352. The gene for *tbp2* was amplified using primers designed for cloning into pAA352 and a band of 1989 bp was found as expected (Figure 5-5A). The DNA was ligated into pAA352 to produce a leukotoxin fusion that was transformed into *E. coli* M15. Transformants were screened by colony blotting (results not shown) and potential positives were further screened by small-scale IPTG induction (Figure 5-5B). The results showed one transformant expressing a protein, however the protein was 90 kD and the fusion of leukotoxin and Tbp2 should yield a protein of about 180 kD. Amplification of *tbp2* with primers designed specifically for pMAL-c5X also resulted in bands of approximately 1989 bp (Figure 5-5C). This new insert DNA was ligated into pMAL-c5X and the ligation transformed into *E. coli* ER2523 and BL21pLys. The transformants were screened using colony blots (results not shown) and any possible positives were further screened using IPTG induction (Figure 5-5D) which yielded no positive results.

Transformants containing insert DNA for *tbp2* were not successful using either pAA352 or pMAL-c5X; therefore, it was decided that in order to include Tbp2 in future animal trials, it was necessary to purify it from a previous construct. Decades ago, *tbp2* from strain HS25 was cloned into pGH433 and this was saved as a glycerol stock. The homology between AVI1 Tbp2 and HS25 Tbp2 could not be determined due to the incomplete sequence of AVI1. However, it

was previously determined that AVI1 was between the old isolates, including HS25, and the new isolates. AVI1 belongs to neither group and therefore it can be assumed that it is the closest to HS25 of the new isolates. Transferrin-binding protein 2 was purified using this construct HS25_Tbp2 or inclusion bodies, which resulted in large amounts of purified Tbp2 (Figure 5-6). Thus, the gene coding for Tbp2 was unable to be cloned using either maltose fusion or leukotoxin fusion methods. Therefore, it was purified from an existing construct by aggregate preparations.

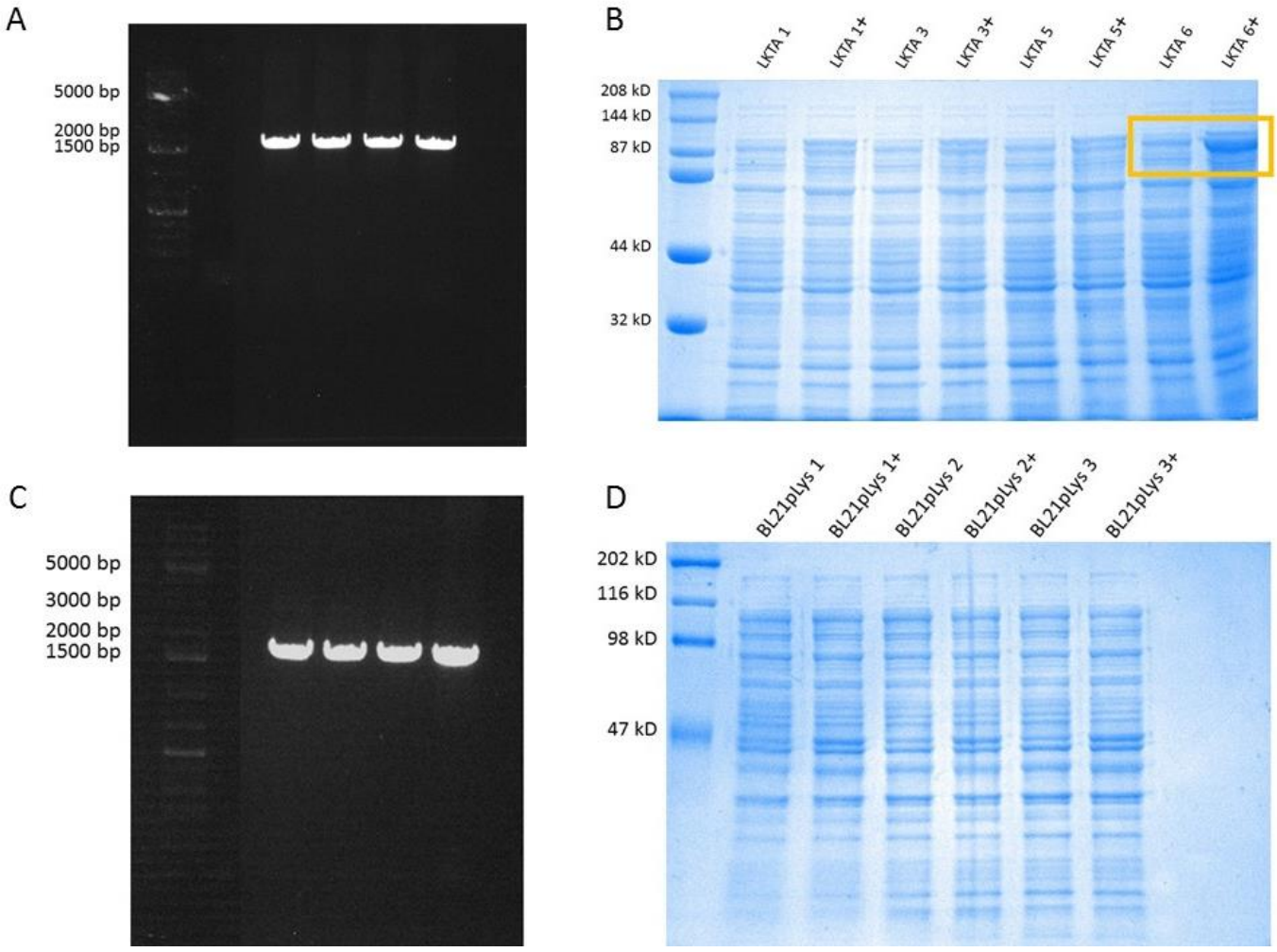


Figure 5-5 PCR and small-scale IPTG induction of Tbp2. (A) Amplification of Tbp2 from the *H. somni* strain AVI1 using primers designed for ligation with vector pAA352. (B) Small-scale IPTG induction of colonies 1 through 6 that were transformed with pAA352/Tbp2 ligation. One tranformant showed expression of an incorrectly sized protein (87 kD). Induced cultures indicated with '+'. (C) Amplification of Tbp2 from the *H. somni* strain AVI1 using primers designed for ligation with vector pMAL-c5X. (D) Small-scale IPTG induction of clones 1-3 transformed with pMAL-c5X/Tbp2 ligation. No protein expression was induced. Induced cultures indicated with '+'.

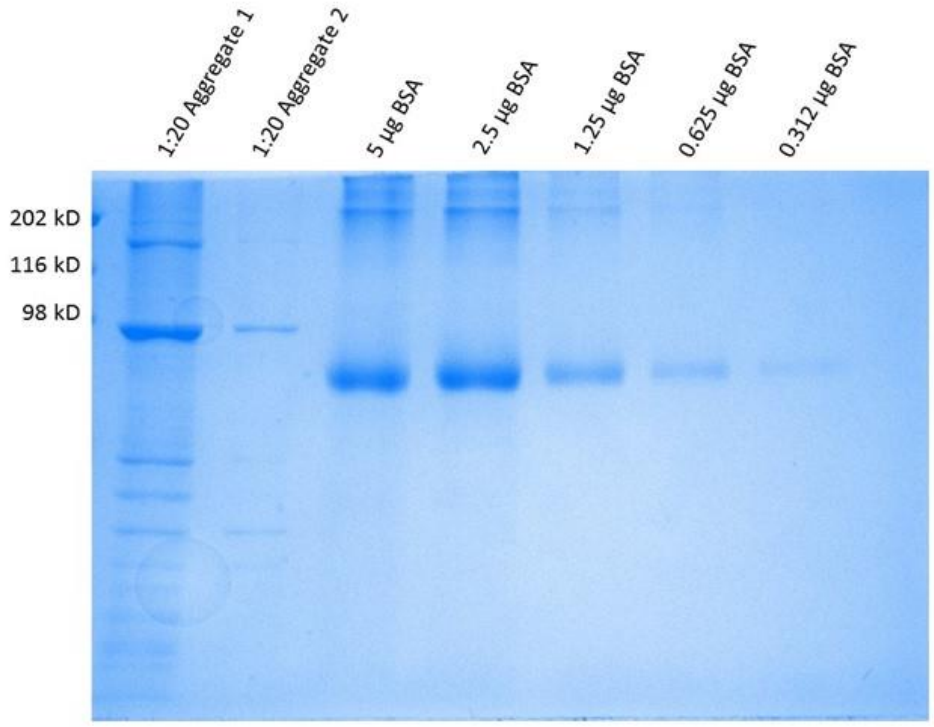


Figure 5-6 SDS-PAGE gel of purified Tbp2. SDS-PAGE gel of aggregates 1 and 2 containing purified Tbp2. Densitometry used serially diluted BSA standards to determine the concentration of protein in each aggregate preparation.

5.4 Receptor 1372 induces both humoral and cellular immune responses in cattle

5.4.1 Proliferation responses following immunization with Receptor 1372

In order to determine the immune responses induced by receptor 1372, the proliferative response of isolated bovine PBMCs was analyzed. Samples of blood were taken from each calf and PBMCs isolated as previously described. Isolated PBMCs were stained and stimulated with Receptor 1372 in order to measure the proliferative response by flow cytometry.

In order to determine if Receptor 1372 induced a cellular immune response, a group of 16 calves containing 8 vaccinates and 8 controls, were immunized with 100 μg of purified Receptor 1372. On day 0, no significant difference between immunized and non-immunized calves was observed. This was as expected given that none of the animals had been exposed to the vaccine or challenge. Figure 5-7B shows that the proliferation of PBMCs isolated before challenge was significantly increased when animals were immunized with Receptor 1372. The PBMCs which were stimulated with only 1 ng and 2 ng of receptor 1372 showed no significant increase while the cells stimulated with 10, 20 and 30 ng did. These results showed a treatment effect when the concentration of antigen was increased for each re-stimulation while also showing that a 10, 20 or 30 ng treatment was sufficient for re-stimulation. Figure 5-7C shows the results of PBMCs isolated 2 weeks post-challenge where no significant differences were observed between immunized and non-immunized animals. From these proliferation assays, it was possible to conclude that the Receptor 1372 was inducing a strong cellular immune response, which also showed a treatment effect.

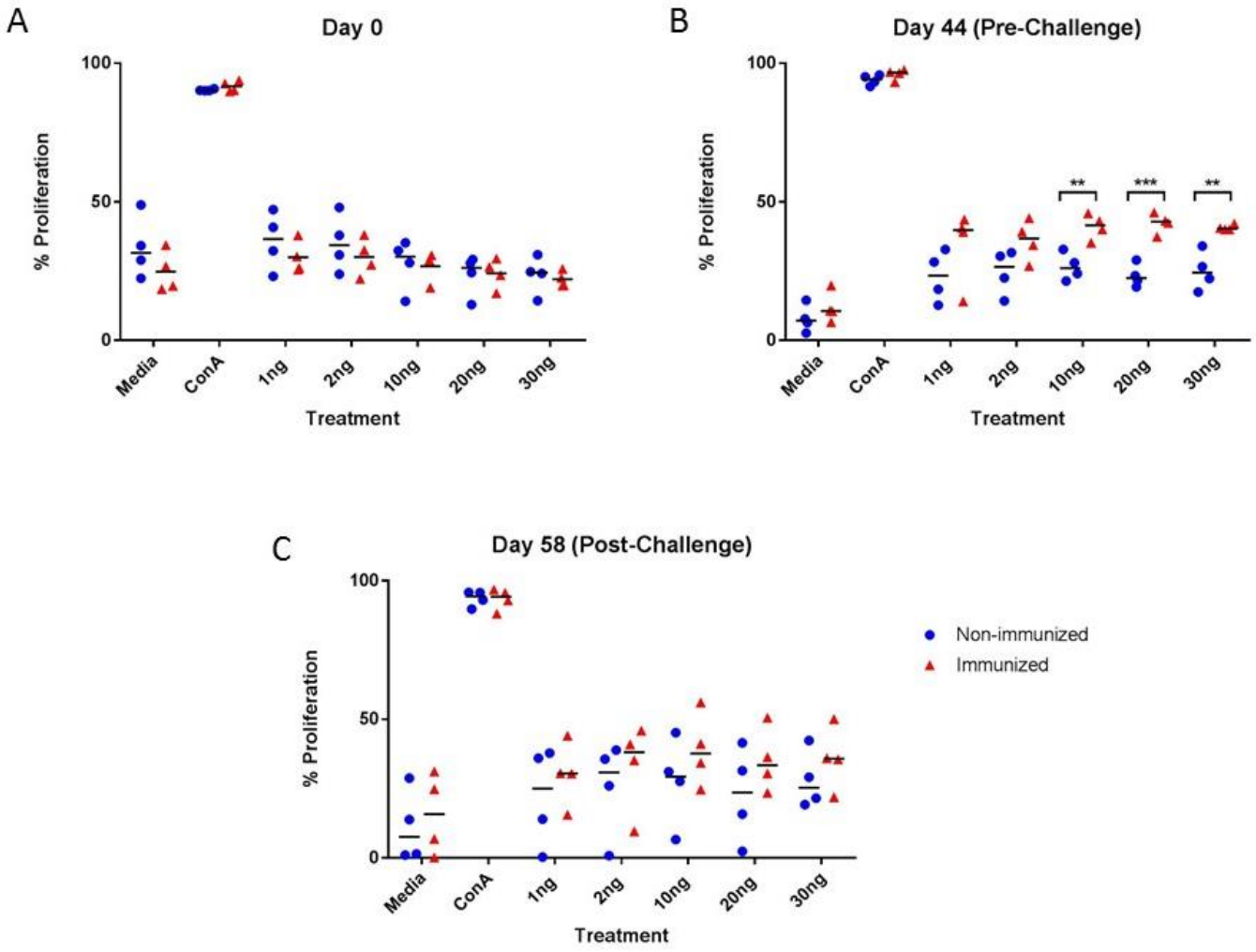


Figure 5-7 Proliferation of PBMCs from calves immunized with Receptor 1372. Calves were immunized with Receptor 1372 and PBMCs were isolated on Day 0, Day 44 and Day 58. (A) Day 0 PBMC proliferation before immunization. (B) Day 44 (Pre-Challenge) PBMC proliferation 2 weeks after calves received a boost immunization. (C) Day 58 (Post-Challenge) PBMC proliferation 2 weeks after calves received an intravenous challenge of *H. somni* AVI1. Significance between non-immunized and immunized were determined by parametric t-test (*=P<0.05, **=P≤0.01, ***=P≤0.001).

5.4.2 Humoral responses following immunization with Receptor 1372

In order to determine if Receptor 1372 induces a humoral response in immunized calves the antibody titres were analyzed by serum ELISA. Blood samples were taken from each calf in order to isolate the serum, which was tested for the presence of antibodies specific for Receptor 1372. The optical densities (ODs) obtained from ELISA were used to determine serum titres against Receptor 1372 in each animal, and these are shown in Figure 5-8. The antibody titres to Receptor 1372 were significantly increased once the immunized animals received both the first immunization and the boost. The titres of Day 44 (Pre-challenge) and day 58 (Post-challenge) were significantly higher in the animals immunized compared to the non-immunized animals. These results show that Receptor 1372 induces a humoral immune response in cattle. Along with the proliferation assays, the serum titres also show that the receptor is immunogenic.

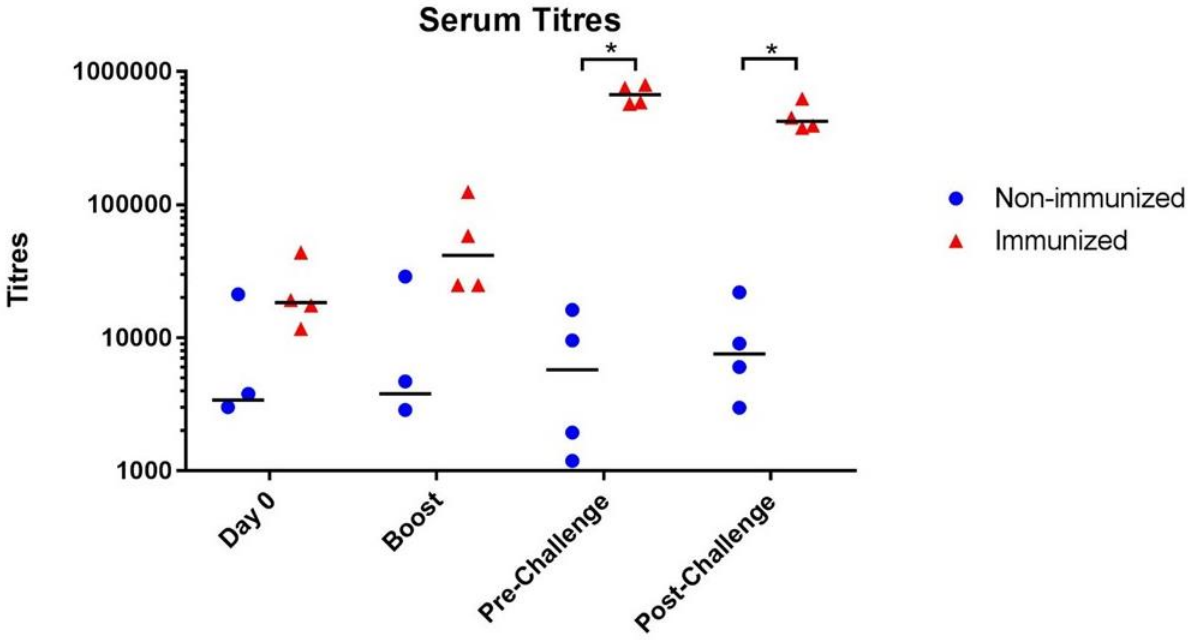


Figure 5-8 Serum titres from calves immunized with Receptor 1372. Calves were immunized with Receptor 1372 and serum samples taken from both immunized and non-immunized animals on Day 0, Boost (day 30), Pre-Challenge (day 44) and Post-Challenge (day 58). Significance between non-immunized and immunized were determined by Mann-Whitney test (*=P<0.05, **=P≤0.01, ***=P≤0.001).

5.5 Tbp2 induces an increase in IFNγ production in mice

5.5.1 Quantitative-PCR of IFNγ and IL-4 levels in murine splenocyte RNA

In order to determine if Receptor 1372, Tbp1 and/or Tbp2 induces an increase in the humoral or cellular immune response in mice, splenocytes were isolated from mice that were immunized with Receptor 1372, Tbp1 and Tbp2. Once isolated, the splenocytes were stimulated with each protein individually before RNA was isolated. With isolated murine RNA, the cellular response was analyzed by expression of IFNγ and the humoral response by expression of IL-4. Expression of these cytokines was analyzed by qPCR. The results of qPCR as seen in Figure 5-9A showed that expression of IL-4 did not change significantly once mice were immunized with the receptor 1372, Tbp1 and Tbp2. While there was no significant increase in IL-4 production, there was down-regulation of IL-4 when ConA was present. As seen in Figure 5-9B, there was no significant increase in production of IFNγ until the last day when animals had received a boost vaccination containing receptor 1372, Tbp1 and Tbp2. On day 28, production of IFNγ increased significantly in the presence of Tbp2. The results of qPCR showed that Tbp2 was inducing an increase in the production of IFNγ and therefore inducing the cellular immune response. There was no detectable change in the humoral response through analysis of IL-4.

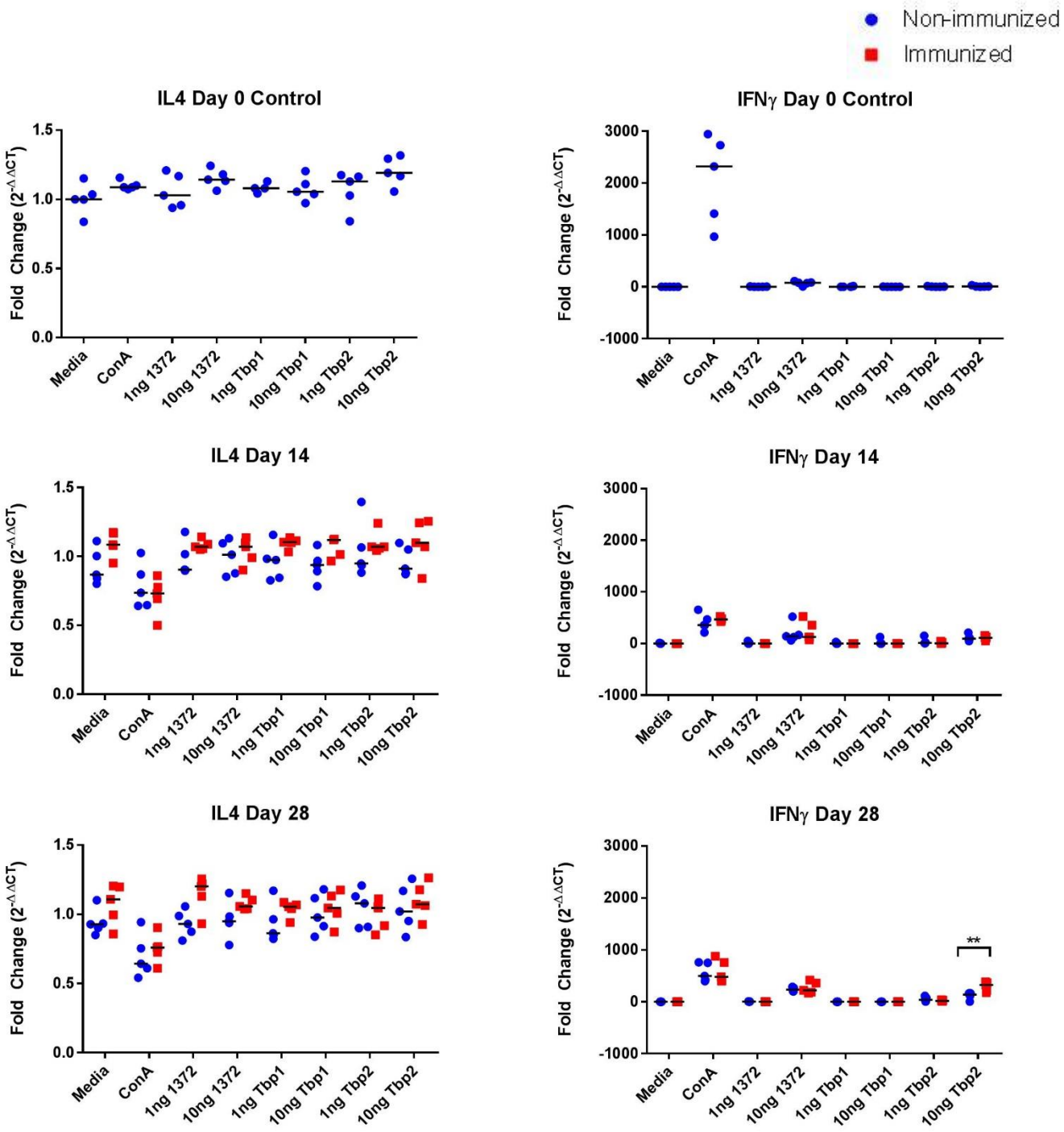


Figure 5-9 Quantitative PCR of IL-4 and IFNγ expression from murine splenocyte RNA. Mice were immunized with receptor 1372, Tbp1 and Tbp2. Splenocytes from immunized and non-immunized mice were isolated and stimulated on day 0, day 14 and day 28. Quantitative PCR measured the fold change ($2^{-\Delta\Delta CT}$) of IL-4 and IFNγ expression at each time point. (A) Expression of IL-4 showing the effect of immunization on the humoral response for day 0, day 14 and day 28. (B) Expression of IFNγ showing the effect of immunization on the cellular immune response for day 0, day 14 and day 28. Significance between non-immunized and immunized were determined by two-way ANOVA with Sidak's multiple comparison test (*=P<0.05, **=P≤0.01, ***=P≤0.001).

5.5.2 Expression of cytokines IFNγ and IL-4 confirmed by ELISA

In order to confirm the results of qPCR discussed above, cytokine ELISA analyzed the presence of both IFNγ and IL-4. When stimulating splenocytes with Receptor 1372, Tbp1 and Tbp2, the cells produced cytokines that were released into the media or supernatant. This supernatant was used for ELISAs that detect the presence of various cytokines. Both IFNγ and IL-4 were observed however, the IL-4 results were inconclusive. In order to evaluate the humoral response, IL-5, another cytokine involved in the Th1 response was analyzed. The results of cytokine ELISA (Figure 5-10) were used to confirm the qPCR results previously discussed. The amount of IFNγ expressed by re-stimulated splenocytes increased in the presence of Tbp2 (Figure 5-10) which was also seen in the results of qPCR. The amount of IL-4 did not change in the animals vaccinated (data not shown) so in order to analyze the humoral response, the amount of IL-5 was analyzed instead for both vaccinated and control animals (data not shown). The results of cytokine ELISAs showed that the amount of IL-5, i.e. the humoral response, remained unchanged between both groups while the amount of IFNγ did increase in the presence of Tbp2. These results, along with the results of qPCR, show Tbp2 as the only protein of the three capable of inducing an increasein the level of IFNγ production.

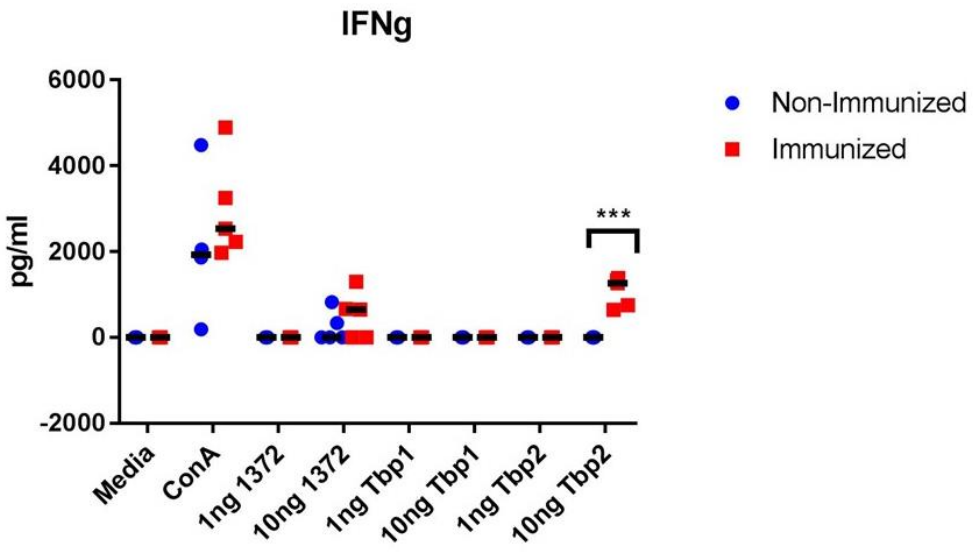


Figure 5-10 Cytokine ELISA of IL-4 and IFNγ expression from murine stimulated splenocyte supernatant. Mice were immunized with receptor 1372, Tbp1 and Tbp2. Splenocytes from immunized and non-immunized mice were isolated and stimulated for 20 hours with 1 and 10 ng treatments each of Receptor 1372, Tbp1 and Tbp2. Analysis of cytokines IL-4 (data not shown), IL-5 (data not shown) and IFNγ for Day 28 supernatant from murine splenocytes confirmed results of qPCR. Results of IFNγ assay of vaccinated and control animals. Significance between non-immunized and immunized were determined by unpaired t-test (*=P<0.05, **=P≤0.01, ***=P≤0.001).

5.6 Receptor 1372, Tbp1 and Tbp2 induce both humoral and cellular immune responses in cattle

5.6.1 Proliferation responses following immunization with Receptor 1372, Tbp1 and Tbp2

In order to determine the immune responses induced by receptor 1372, Tbp1 and Tbp2, I analyzed the proliferative response of isolated bovine PBMCs by applying the same techniques as the previous proliferation assays. It was expected that on day 0, PBMCs would not show a significant increase in proliferative response to receptor 1372, Tbp1 and Tbp2 however, Figure 5-11A showed that there was a significant difference between immunized and non-immunized animals for PBMCs stimulated with media, Receptor 1372, Tbp1 and Tbp2. As seen in Figure 5-11B, the PBMCs that were isolated on the day of boost showed an increase in proliferation only with media and Tbp1. On day 44 (Figure 5-11C), PBMCs stimulated with Tbp1 and Tbp2 showed a significant proliferative response as expected. It was also expected that Receptor 1372 would result in an increase in proliferation but this was not the case. In Figure 5-11D, it was observed that none of the treatments resulted in a significant increase in proliferation when animals were immunized. These results showed that Receptor 1372, Tbp1 and Tbp2 induced an increase in the cellular immune response by causing an increase in PBMC proliferation, which means these proteins are immunogenic. However, it is difficult to say that these results are biologically significant since the trend is not obvious.

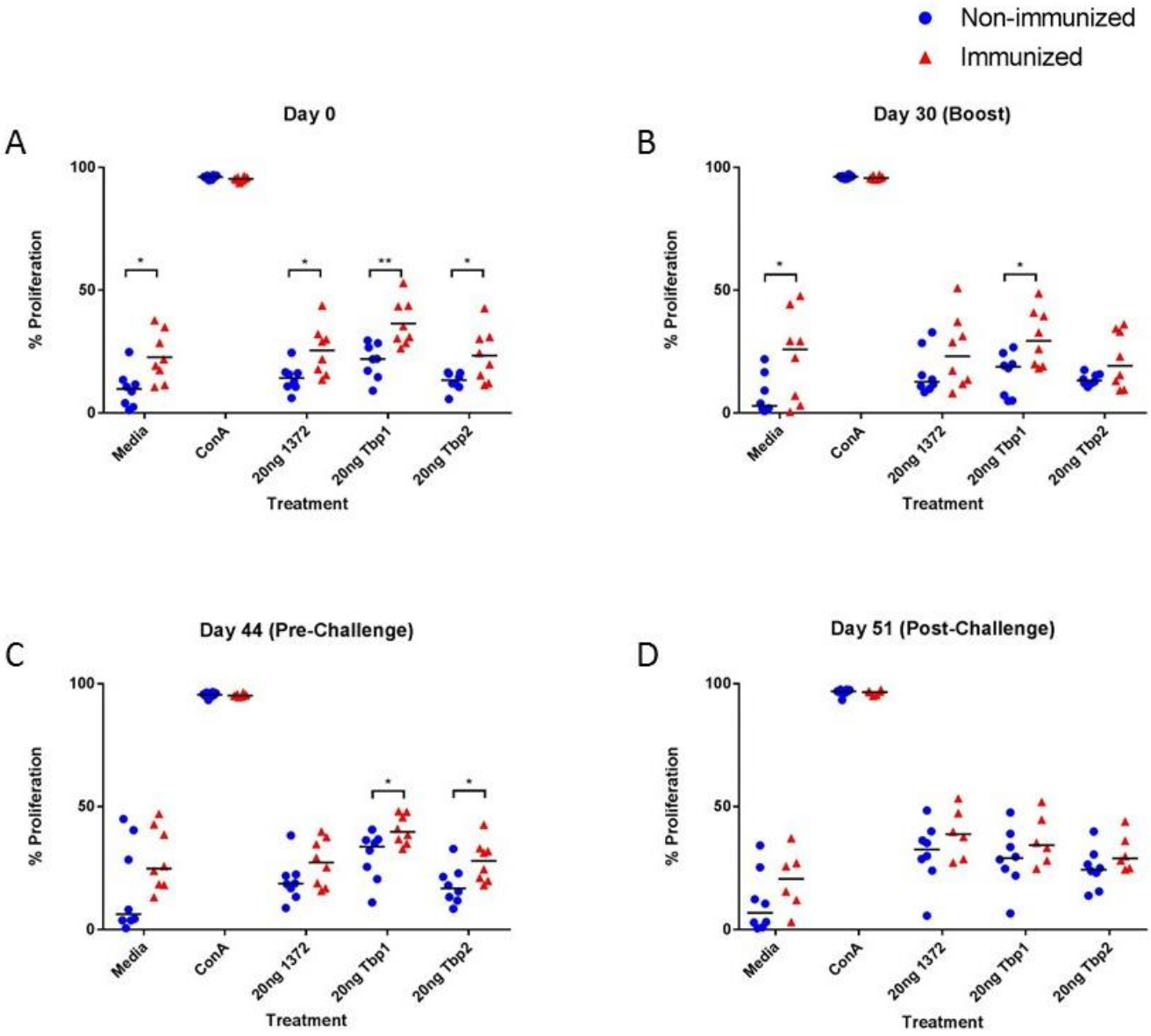


Figure 5-11 Proliferation of PBMCs from calves immunized with Receptor 1372, Tbp1 and Tbp2. Calves were immunized with Receptor 1372, Tbp1 and Tbp2. PBMCs were isolated on Day 0, Day 44 and Day 58. (A) Day 0 PBMC proliferation before immunization. (B) Day 30 (Boost) when calves received a boost immunization. (C) Day 44 (Pre-Challenge) PBMC proliferation 2 weeks after calves received a boost. (D) Day 58 (Post-Challenge) PBMC proliferation 2 weeks after calves received an intravenous challenge of *H. somni* AVI1. Significance between non-immunized and immunized were determined by parametric t-test (*=P<0.05, **=P≤0.01, ***=P≤0.001).

5.6.2 Humoral responses following immunization with Receptor 1372, Tbp1 and Tbp2

In order to determine if Receptor 1372, Tbp1 and Tbp2 induce a humoral immune response, the antibody titres were analyzed by serum ELISA. Serum was obtained from each animal as described above. The serum was used to determine the presence of antibodies specific to each protein. Antibody titres for each animal did not show an increase in antibody titres when exposed to Tbp1 (Figure 5-12A). This was as expected since Tbp1 is known to cause more of a cellular response while Tbp2 is known to cause more of a humoral response. A small increase in titres for immunized animals was observed though it was insignificant. Antibody titres to Tbp2 showed a significant increase when animals were immunized with the three proteins (Figure 5-12B). As seen in Figure 5-12C, there was not a significant increase in antibody response to Receptor 1372 in immunized animals that was expected based on the previous cattle trial. The antibody titres show that Tbp2 induces the humoral immune response, while Tbp1 and Receptor 1372 do not. The results of ELISA and proliferation assays confirm that Tbp1 causes more of a cellular response while Tbp2 causes a stronger humoral response. Receptor 1372 was shown to be immunogenic in the proliferation results however, these results and the antibody titres do not agree with the previous trial.

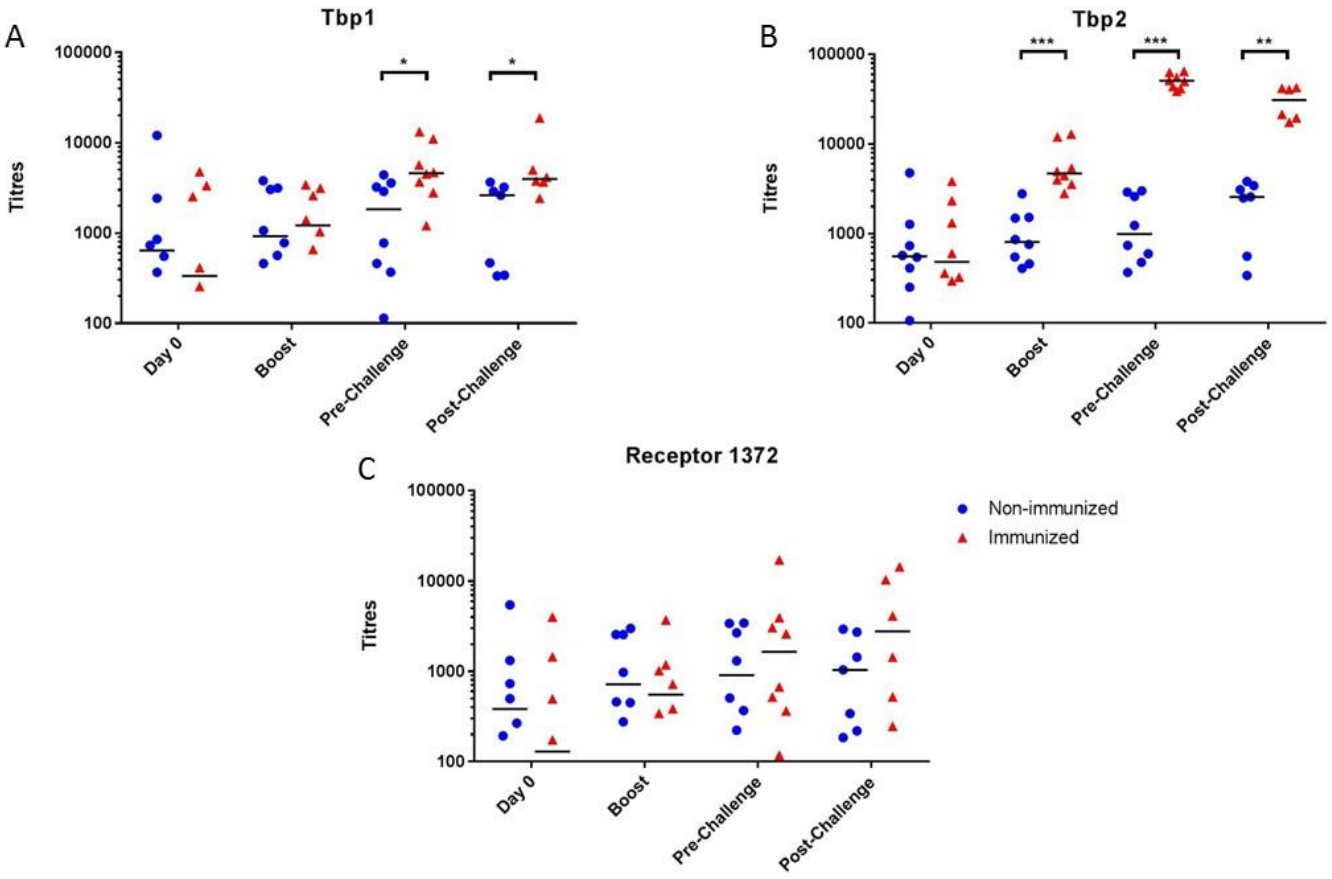


Figure 5-12 Serum titres from calves immunized with Receptor 1372, Tbp1 and Tbp2. Calves were immunized with Receptor 1372, Tbp1 and Tbp2. Serum samples were taken from immunized and non-immunized animals on Day 0, Boost (day 30), Pre-Challenge (day 44) and Post-Challenge (day 58). (A) Serum titres of antibodies specific for Tbp1. (B) Serum titres of antibodies specific for Tbp2. (C) Serum titres of antibodies specific for Receptor 1372. Significance between non-immunized and immunized were determined by Mann-Whitney test (*=P<0.05, **=P≤0.01, ***=P≤0.001).

6.0 GENERAL DISCUSSION AND CONCLUSIONS

The BRD complex is an economically important disease caused by a number of agents such as viral and bacterial pathogens including *H. somni* [1-3]. *H. somni* causes both local and systemic infection with the incidence of systemic infection increasing over the past few decades. I hypothesized that vaccination with TonB-dependent proteins produced by *H. somni* would be protective against experimental infection and that a good cellular immune response is important for protection. Currently available vaccines that target *H. somni* are composed of killed whole cells using strains that were isolated decades ago. One exception is a bacterial extract vaccine consisting of *in-vivo* expressed antigens such as the IRPs. Based on this knowledge, the objectives of this study were to clone and express the genes coding for three TonB-dependent proteins and examine their effect on the humoral and cellular immune responses of cattle during experimental infection with *H. somni*. During this study, the work accomplished included: purification of Receptor 1372, Tbp1 and Tbp2, vaccination of cattle with Receptor 1372, characterisation of the humoral and cellular immune responses induced, vaccination of mice with Receptor 1372, Tbp1 and Tbp2 to determine if one or all of the proteins induce a cellular immune response and finally characterization of the immune responses and protective capacity in cattle of a vaccine containing Receptor 1372, Tbp1 and Tbp2.

Receptor 1372, a heme/hemoglobin receptor family protein, is similar to the IRPs found in *M. haemolytica*, *P. multocida* and *H. influenza* [52-55]. Other than being a receptor and an iron-regulated protein, the specific function of this receptor remains unclear and no examples of similar receptors were found. Receptor 1372 was cloned, expressed and purified using the vector pGH433His and *E. coli* DH5αF'IQ. Once purified, I studied the cellular and humoral immune responses induced in mice followed by cattle. In mice, Receptor 1372 induced neither an increase

in IL-4, IL-5, or IFNγ production. In cattle, this protein was first tested by immunization with Receptor 1372 alone. The results of the cattle trial suggested that the receptor was immunogenic, inducing a strong increase in antibody production as well as a strong increase in PBMC proliferation. However, while combined with Tbp1 and Tbp2 in a subunit vaccine, Receptor 1372 did not induce a cellular or a humoral response. This may have been due to the presence of Tbp1 and/or Tbp2 interfering with the interaction of the receptor with host immune cells [79-81]. From this study, I was able to show that Receptor 1372 is capable of inducing both a cellular and a humoral immune response in cattle when it is the sole component of a vaccine. The fact that Receptor 1372 is iron-regulated and present in larger amounts when the bacterium is in an iron-depleted environment, suggests that Receptor 1372 could be a candidate vaccine antigen against *H. somni* infection in cattle. Unfortunately, I was unable to determine if this receptor would be protective, as it would need further study with a second cattle trial. However, I am able to conclude that this IRP is immunogenic and immunization with Receptor 1372 results in an increase in PBMC proliferation as well as an increase in the production of Receptor 1372-specific antibodies.

Transferrin and lactoferrin-binding proteins have been studied in *Neisseriaceae*, *Moraxellaceae* and *Pasterellaceae* such as *Pasteurella multocida*, *Haemophilus influenzae*, *Neisseria gonnorrhoeae*, *Actinobacillus pleuropneumoniae* and *Neisseria meningitides* [52-63]. Transferrin-binding protein 1 is equivalent to *A. pleuropneumoniae* and *M. haemolytica*'s, TbpA discussed previously. TbpA and Tbp1 are iron-regulated outer membrane receptor proteins involved in the uptake of iron [52-55]. These proteins tend to be expressed in larger amounts when in an environment depleted of iron making them good candidates to study the immune responses induced by *H. somni* antigens [65]. Previously, the use of TbpA from *M. haemolytica* and *H. somni*

in vaccines did not show evidence of an antibody response [63, 64]. It was hypothesized by Potter *et al*. that this may have been due to TbpA inducing primarily a cellular immune response [63].

The *tbp1* gene was cloned and Tbp1 expressed and purified using the MBP fusion vector, pMAL-c5X and *E. coli* ER2523. With the purified Tbp1, the immune responses induced were first studied in mice. In mice, immunization with Tbp1 did not result in an increase in production of humoral or cellular cytokines, IL-4/IL-5 and IFNγ, respectively. Next, the immunogenicity in cattle was tested by immunizing with a subunit vaccine containing Receptor 1372, Tbp1 and Tbp2. From this trial, I found that Tbp1 has the potential to be protective however; it may only be an ideal candidate for a vaccine if the goal is to induce a stronger cellular immune response. I was able to determine that Tbp1 does induce a significant cellular immune response shown by the increase in PBMC proliferation but it does not appear to have much impact on the humoral response, which agrees with the other studies of *H. somni*'s Tbp1 and *M. haemolytica*'s TbpA. Whether there is interference between Receptor 1372, Tbp1 and Tbp2 causing the low humoral response would need to be further evaluated. If a stronger cellular response were desired, Tbp1 would be an ideal candidate vaccine antigen to include among others that induce a strong antibody response. This would ensure that the vaccine is stimulating two very important aspects of adaptive immunity. Since *H. somni* is an intracellular pathogen, it may be important to ensure cell-mediated immunity is primed while also priming for an antibody response to combat it before and after it enters immune cells.

Transferrin-binding protein 2 is similar to TbpB of *A. pleuropneumoniae* and *M. haemolytica*, in that it is a co-factor to Tbp1, the main component of the iron-transport system in many species including *H. influenza*, *P. multocida*, *M. haemolytica* and *Neisseria* species [52-55]. As with Tbp1, Tbp2 has also been studied as a potential vaccine antigen. Previously, it was shown

that Tbp2 from *H. somni* induces a strong antibody response when used in the immunization of cattle [69]. It was also found that TbpB from *M. haemolytica* and many other species of bacteria induces a protective immune response and lessens the degree of disease, making it an ideal vaccine candidate [63].

As with Receptor 1372 and Tbp1, *tbp2* was cloned and the protein expressed in various vectors and *E. coli* strains. When this was unsuccessful, Tbp2 was purified from a decades-old strain of *H. somni*, HS25. Previously, it had been cloned using the pGH433 vector and Tbp2 was purified by aggregate preparations. Once purified, the immune responses induced by Tbp2 were examined in mice, where Tbp2 was the only protein capable of inducing a cellular immune response observed by the increase in production of IFNγ. Once it was established that a response could be induced in mice by one of the three proteins, Tbp2 was combined in a subunit vaccine with Receptor 1372 and Tbp1 and used for the immunization of cattle. In cattle, it was found that Tbp2 induced a stronger humoral response than it did a cellular response. This finding agrees with other studies of a similar nature testing the protective capacity of Tbp2. Since Tbp2 is capable of inducing such a strong humoral response, it is a great candidate for vaccine use. From this study, I was able to conclude that Tbp2 is immunogenic in mice and cattle, inducing a stronger cellular response in mice and a stronger humoral response in cattle. The protective capacity of this protein would need to be studied further, perhaps on its own in a vaccine.

Together, Tbp1 and Tbp2 play a central role in the pathogenesis of many species as described by Cornelissen et al. when they showed that a mutant *N. gonorrhoeae* deficient in a transferrin receptor was avirulent in humans [63, 82]. These transferrin-binding proteins have been shown to be protective antigens in some species including *H. somni* and *M. haemolytica* [63, 69].

It is important to induce an antibody response when looking for a vaccine however, it may be time to consider the importance of a cellular immune response especially for intracellular pathogens. Since antibodies are useful in neutralizing and controlling extracellular pathogens, it is natural that this would be our main goal. However, *H. somni* has been found to survive and even impair the activities of multiple immune cells and this may indicate that it is necessary to look more closely at what cell-mediated immunity can do in the case of an *H. somni* infection. A vaccine capable of inducing a strong antibody response as well as inducing a protective cellular response may be important in combatting histophilosis in the cattle industry. The importance of the cellular immune response and the characterization of the cellular immune response to other *H. somni* antigens would need to be further studied in order to understand more about this disease.

In conclusion, based on the results of the murine trial and both cattle trials, Receptor 1372, Tbp1 and Tbp2 proved to be immunogenic in cattle as they induced either a cellular or a humoral response. In the case of Receptor 1372, it was capable of inducing both a strong cellular and a strong humoral response in cattle, suggesting it may be a good candidate vaccine antigen. In cattle, Tbp1 and Tbp2 were capable of inducing either a stronger humoral response or a stronger cellular response respectively and this may need to be taken into consideration when selecting vaccine candidates. In mice, only Tbp2 proved to be immunogenic by inducing a significant increase in the cellular immune response. Whether these proteins are protective would need to be studied further with a trial involving the immunization of cattle with Receptor 1372, Tbp1 and Tbp2.

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