Discovery and Evaluation of Immunogenic Antigens for Bovine Brucellosis Serodiagnostics

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Preliminary Content

Abstract

Brucella spp. are zoonotic infectious agents, primarily of livestock, that cause the disease brucellosis. Bovine brucellosis, caused by *B. abortus,* is of greatest concern due to the disease's significant economical and public health impact. Canada fully eradicated bovine brucellosis from domesticated cattle herds in 1985, however, continued surveillance through screening for *B. abortus* exposure is paramount to the maintenance of bovine brucellosis eradication nationwide. The Canadian Food Inspection Agency (CFIA) is responsible for the surveillance of bovine brucellosis outbreaks in Canada and the maintenance of eradication. Current *B. abortus* serodiagnostics and serological screening is mostly based on the detection of antibodies against *Brucella* lipopolysaccharide (LPS), a highly immunogenic component of the outer cellular membrane. Such tests face difficulties with false positive results due to cross reactivity with other Gram-negative bacteria that produce LPS. The purpose of the research presented here was to address this issue through identifying new *B. abortus* protein antigens for the improvement of serological test specificity. In this study, 101 candidates were identified through predictive bioinformatic analyses and selected for immunogenic evaluation. While none of the expressed candidates displayed positive serological activity with in-house brucellosis positive bovine serum panels, the workflow presented here can be used for continued research and the assessment of more proteins from *B. abortus* and other bacterial pathogens.

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List of Abbreviations

aa – Length in Amino Acids
ATP – Adenosine Triphosphate
BBATs – Buffered *Brucella* Antigen Tests
BCA – Bicinchoninic Acid
BLASTN – Basic Local Alignment Tool for Nucleotides (NCBI)
BLASTP – Basic Local Alignment Tool for Proteins (NCBI)
bp – DNA Base Pair
BPAT – Buffered Plate Agglutination Test
BSS – Bovine Surveillance System
BST – Brucellin Skin Test
bv. – Biovar
C-ELISA – Competitive Enzyme-Linked Immunosorbent Assays
CDS – DNA Coding Sequence
CE – Cellular Envelope
CFIA – Canadian Food Inspection Agency
CFT – Complement Fixation Test
CFU – Colony Forming Unit
CSFV – Classical Swine Fever Virus
dATP – Deoxyadenosine Triphosphate
dCTP – Deoxycytosine Triphosphate
ddNTP – Dideoxynucleotides Triphosphate
dGTP – Deoxyguanosine Triphosphate
dH_2O – Distilled Water
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribonucleic Acid
dNTP – Deoxynucleotide Triphosphates
dTTP – Deoxythymidine Triphosphate
E Value – Expect Value
ECL – Enhanced Chemiluminescence
EDTA – Ethylenediaminetetraacetic Acid
ELISA – Enzyme-Linked Immunosorbent Assays
EU – European Union
FPA – Fluorescence Polarization Assay
FPSR – False Positive Serological Results
g – Grams
GC content – Guanine-Cytosine Content
GFP – Green Fluorescent Protein
GRDI – Genomics Research and Development Initiative
HCl – Hydrochloric Acid

His – Histidine
HRP – Horseradish Peroxidase
I-ELISA – Indirect Enzyme-Linked Immunosorbent Assays
ID – Identification
IgG – Immunoglobulin G
IPG – Identical Protein Groups
kb – DNA Kilobase Pair
L – Litre
LB – Luria-Bertani
LPS – Lipopolysaccharide
M – Molar
mAb – Monoclonal Antibody
Mbp – DNA Mega Base Pair
MCS – Multiple Cloning Site
mg – Milligram
MHC – Major Histocompatibility Complex
mL – Millilitre
mM – Millimolar
MQH_2O – Milli-Q Purified Water
mRNA – Messenger Ribonucleic Acid
NCBI – National Center of Biotechnology Information (USA)
spp. – Species
ng – Nanograms
Ni-NTA –Nickel Nitrilotriacetate
nm – Nanometers
nr database – Non-Redundant Protein Database (NCBI)
nr/nt database – Non-Redundant Nucleotide Database (NCBI)
nt – Nucleotide
OD – Optical Density
OIE – World Organisation for Animal Health
OMP – Outer Membrane Protein
OPS – O-chain Antigenic Polysaccharide
ORF – Open Reading Frame
Ori – Replication Origin
PAMPs – Pathogen Associated Molecular Patterns
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
RBC – Red Blood Cell
RefSeq – Reference Sequence (NCBI)
R-LPS – Rough Lipopolysaccharide

RNA – Ribonucleic Acid
rOMP – Recombinant Outer Membrane Protein
rpm – Revolutions per Minute
RT – Room Temperature
RTS – Rapid Translation System
SAT – Serum Agglutination Test
SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLF – Sequential Lateral Flow
S-LPS – Smooth Lipopolysaccharide
sMQH2O – Milli-Q Purified Water, Sterilized by Autoclave
str. – Bacterial Strain
T4SS – Type 4 Secretion System
T7P – T7 Promoter
T7T – T7 Terminator
USA – United States of America
UV – Ultra Violet
V – Volts
WEB – Wash/Equilibrium Buffer
WGS – Whole Genome Sequencing
WS – Qubit Working Solution
^{x} g – Times Gravity
µg – Microgram
µm – Micrometer
µM – Micromolar
µL – Microlitre

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1. Introduction

1.1 Preface

Brucella spp. are zoonotic infectious agents, primarily of livestock, that cause the disease brucellosis. Bovine brucellosis, caused by *B. abortus,* is of greatest concern due to the disease's significant economical and public health impact. Bovine brucellosis in Canada was eradicated in all domestic cattle herds by 1985. Nevertheless, sporadic cases are still possible within those populations due to the continued prevalence of the disease-causing agent *B. abortus* in wildlife reserves as well as in other parts of the world. The CFIA is currently the branch of the Canadian government responsible for monitoring and managing any bovine brucellosis endemics. Although no outbreaks in domestic cattle in Canada have been reported since 1986, constant surveillance is essential for maintenance of Canada's brucellosis free status. Additionally, the improvement of brucellosis surveillance methods is an ongoing objective of the Canadian government. The Canadian government's Genomics Research and Development Initiative (GRDI) has provided funding for accomplishing that task, which included the funding for the work presented in this thesis.

Previous work on the identification of *B. abortus* protein antigens was performed by a former MSc student in the CFIA research group (1). That preceding research encompassed preliminary testing of 100 candidates encoded by the *B. abortus* genome, the majority of which are predicted to be secreted/extracellular and outer membrane localized (1: section 4.2.2). Those 100 candidates were not included for evaluation in this study, and 10 of them were shown to be antigenic and had the potential for diagnostic applications (1). Differences between that study and the work presented in this thesis are that in the previous study expressed candidates were not purified nor quantified prior to serum reactivity testing, volumes and dilutions of serum and

secondary antibodies were not optimized for dot blot assays, and chemiluminescence was not applied for imaging immunoreactivity reactions in real-time. Additionally, in the previous study the same challenges with *in vitro* expression were not experienced and the majority of the protein candidates tested were predicted to be secreted/extracellular or outer membrane localized rather than periplasmic localized as was the case in this study. Remaining protein candidates encoded by the *B. abortus* genome, that are predicted to be secreted/extracellular and outer membrane localized and had not been previously tested, were investigated for diagnostic potential in this research with the addition of those localized in other portions of the cellular envelope, namely, the periplasm and cytoplasmic membrane. Candidate selection and evaluation protocols implemented in this study were also used in the previous project on *B. abortus* as well as for multiple bacterial organisms studied by the CFIA research group. These bacteria included *Mycobacterium bovis* and *Listeria monocytogenes*. The scope of this thesis is to analyze and characterize the immunogenicity of selected extracellular and cellular envelope localized protein candidates encoded by the *B. abortus* str. 86/8/59 reference genome. By searching for potential protein candidates through bioinformatic analysis of the *B. abortus* genome sequence, followed by experimental assessment of their immunogenicity, this research has expanded a similar study conducted recently by our group to discover novel antigens for the improvement of bovine brucellosis serodiagnostics in the future.

1.2 Literature Review

I. Bovine Brucellosis: Causation, Disease, and Diagnostics

i. Genus *Brucella* and Features of the *Brucella* Genome

Brucella is a pathogenic genus of bacteria, in the class alphaproteobacteria, that causes the contagious and chronic zoonotic disease brucellosis (2). *Brucella* spp. are Gram-negative, small, non-motile, non-spore forming coccobacillus that facultatively reside in the intracellular niche of mammalian phagocytic cells, but can also persist in the soil and pathogenize certain plants (2, 3, 4). Individual brucellae cells are 0.6 – 1.5 μm long, between 0.5 and 1 μm wide, and are usually found as singlets (5). Members of the *Brucella* genus have two circular chromosomes that are 2.05 Mbp and 1.15 Mbp in size (6, 7) and together have been demonstrated to contain more than 3000 Open Reading Frames (ORFs) (8, 9). *Brucella* spp. have genomic GC contents under 60% (10, 11) and the two chromosomes of *Brucella* spp. have highly similar GC contents to one another. Chromosome 1 is similar in structure and function to other bacterial genomes, whereas chromosome 2 has characteristics similar to plasmids but is much larger (12). The major designated species within the genus are recognized according to their host infectivity; *B. abortu*s, *B. melitensis*, *B. suis*, *B. canis*, *B. neotomae*, and *B. ovis* (13) are the etiological agents of brucellosis in bovine, both caprine and ovine, porcine, canine, desert wood rat, and specifically ovine populations, respectively (14, 15). *B. ceti* and *B. pinnipediae* have also been found in marine populations. *B. ovis and B. neotomae* are not documented as pathogenic to humans. There are also very few documented cases of zoonosis from *B. ceti* and *B. pinnipediae*. Furthermore, the main species that are of serious public health concern due to the high risk of zoonotic transmission to humans are *B. abortu*s, *B. melitensis*, *B. suis*, and *B. canis* (14). In the presence of serious *Brucella* infection, massive loss from heard 'abortion storms' has been documented

during large scale overlapping distribution of cattle, goats, swine, and sheep. In mixed animal population cases, where livestock contract brucellosis infections from an animal host of a separate species, there is no documentation of further transmission within the new population following initial species cross-over events (2). This phenomenon is likewise observable following brucellosis zoonosis as documented human-to-human transmission is extremely rare (16, 17).

Clinical manifestations of infection with pathogenic *Brucella* species in humans were originally known as Malta Fever prior to identification of the *Brucella* genus and its various species. Brucellosis represents a serious and ongoing infection in humans (1, 17) with an infectious dose of 10 – 100 cells by aerosol (18). The infectious dose is an alarmingly low value and is one of the primary reasons all pathogenic *Brucella* species are categorized at biosafety level 3. Notwithstanding the fact that infections in humans by some strains can be much more debilitating than others (2). Clinical documentation of brucellosis in humans demonstrates that infection by a member of the *Brucella* genus, in almost all cases, is the result of zoonotic exposure or consumption of contaminated products. However, transference to humans by inhalation and through the skin has also been reported (17, 19). Once infected, the disease causes non-specific acute symptoms including weight loss, fatigue, and general malaise for weeks or months. Chronic infection can result in long-term morbidity as well as persistence and frequent recurrence of the acute symptoms over many years (16, 20, 21). Furthermore, human-to-human transmission is rare but has been documented as transmissible congenitally and through breast feeding, from sexual contact, and from blood transfusions and bone marrow transplants (16).

As a result of the severity of the disease, the frequent failure of clinical identification following exposure, as well as the low infectious dose and the ease of large-scale culturing,

lyophilization, and dispersal of the pathogen, *Brucella* has long been of significance as an agent of bioweaponry and bioterrorism (22, 23). *B. suis* was one of the first bacteria to be weaponized (24). The seriousness of *Brucella* pathogenicity is attributable to its ability to circumvent innate and adaptive immunity (25), as well as, in part, to unique and uncommon virulence factors that are not well known or characterised. *Brucella* species lack common classical virulence factors found in other Gram-negative pathogens. Due to this, there has been limited characterization of the genetics of *Brucella* virulence (9, 26). One of the virulence factors that has been identified and characterized in all *Brucella* species is the Type IV Secretion System (T4SS) (27). The T4SS is composed of large complexes of proteins that traverse the cellular envelope and contain a channel for the translocation of other proteins or protein–DNA complexes. Translocation of molecules by the T4SS is dependent on cytoplasmic ATPases providing the energy for large conformational changes (28).

Brucella species have long caused great economic and public health challenges and continue to be one of the world's major zoonotic problems. There are also no vaccines approved and available for human use (22) and treatment with antibiotics is arduous and often minimally effective once the disease has become an established chronic infection (21, 29). Many countries have been working towards eradication of the more devastating *Brucella* species in recent decades, however, this comes at a high economic cost to implement and maintain (21) as will be discussed in more detail for *B. abortus* in Canada.

ii. *Brucella abortus* and Bovine Brucellosis

B. abortus is an aerobic, Gram-negative coccobacillus which displays virulence towards humans (26). *B. abortus* is of particular importance within the *Brucella* genus due to its significant economic impact as the causative zoonotic agent of the disease bovine brucellosis.

Bovine brucellosis causes devastating regeneration detriment in the form of spontaneous abortions and sterility throughout bovine herds (13, 29, 30, 31). *B. abortus* can quickly spread through cattle herds as well as to humans through direct contact with infected animals, their products, or through the consumption of contaminated food and water (17, 29). Thus, the rapid identification of local *B. abortus* exposure in bovines is of importance both in terms of public health and the Canadian cattle industry.

At this point in time there are over 250 *B. abortus* genome assemblies posted on the National Center for Biotechnology Information (NCBI) GenBank database (10) operated by the U.S. government with many strains present that are representative of each of the *B. abortus* biovars. *B. abortus* evolution has resulted in 8 differentiable biovars, which are 1 – 7 and 9 (32), with biovar 2 being one of the most commonly isolated from humans who have contracted the disease (33). Each biovar has various strains within it and *B. abortus* 86/8/59 is a reference strain for biovar 2 (34). *B. abortus* biovar 2 has also been particularly important in Canada as the first and only outbreak isolate since Canada's eradication of bovine brucellosis in the national domestic cattle herd in 1985 (17, 35). An outbreak in 1986 occurred in Alberta but since then the Canadian government has been continually successful in maintaining its Brucellosis-free status with no outbreaks (29, 35). The Canadian Food Inspection Agency (CFIA) is the department responsible for the management and maintenance of eradication in Canada through surveillance and research into improving screening methodologies. Once *B. abortus* is identified in domestic cattle herds in Canada, all infected and exposed bovines are slaughtered and their owners compensated (29). While the domestic bovine populations in Canada are brucellosis free, wildlife reservoirs still exist in bison and caribou herds in parts of Alberta and the arctic (17). Eradication through slaughter has been the most efficient option for managing the disease in

cattle in Canada. However, vaccination methods do exist which can provide moderate aid in eradication initiatives around the world. For *B. abortus*, vaccines have been developed using strains S19, RB51, 45/20, and SR82 with S19 and RB51 being the most used (5, 36). Universal issues with *Brucella* spp. vaccines include not being completely effective in preventing animals from becoming infected as well as causing interference with diagnostic screening. Differentiating between vaccinated and *B. abortus*-infected bovines requires the development of improved serological techniques. Due to these issues, vaccination of cattle in Canada is prohibited and according to the World Organisation for Animal Health (OIE), a country cannot vaccinate against the disease if it wishes to hold brucellosis-free status (29). Live attenuated *Brucella* vaccines have also been reported to cause animal abortion, infertility, and weak offspring (37). The CFIA operates the Bovine Surveillance System (BSS) which works to ensure the domestic national herd remains brucellosis-free by monitoring internationally traded cattle and through surveillance of abattoirs, slaughterhouses, artificial insemination programs, and imports and exports of bovine products. Positive serological screening results lead to follow up testing, epidemiological investigations, and potentially destruction of infected or exposed bovines and their products (38).

iii. Diagnostic Methods for *Brucella* infection

Bovine brucellosis, also known as undulant fever, is a disease caused by *B. abortus* that can have significant impacts both economically and from a public health perspective (31). As mentioned, bovine brucellosis symptoms include spontaneous abortions and sterility throughout infected cattle herds while infected humans experience recurring infections with symptoms such as fever, weakness, weight loss, and malaise during acute and chronic stages of infection (16, 20, 21). Moreover, bovine brucellosis spreads through contact with infected animals, their products,

and by foodborne transmission (17, 29). The majority of human cases in Canada are a consequence of travel to endemic areas and are caused mainly through the consumption of unpasteurized dairy products from these areas (17). Infection can also occur through exposure to wildlife reserves such as caribou, bison, and aquatic mammals.

Infection by *Brucella* progresses through four steps which are common for intracellular pathogens. These steps are adherence, invasion, establishment, and dissemination within the host (39). *Brucella* cells can infect macrophages and it is hypothesized that antibody or complement mediated phagocytosis is exploited for host cell adherence and invasion. Following macrophage invasion, *Brucella* cells occupy a niche within phagosomes and inhibit phagosome-lysosome fusion via rapid acidification of the niche. *Brucella* has also been found to occupy the endoplasmic reticulum as a location for multiplication (39). Additionally, *Brucella* spp. and *B. abortus* possess non-classical virulence factors compared with other pathogenic bacteria such as *E. coli* (40). This is of particular relevance to brucellosis pathology and host interaction. *Brucella* lipopolysaccharides (LPSs), for example, are much less immunologically active than *E. coli* LPSs. They are also non-endotoxic. This is due to a number of unique molecular differences in the internal bonding of each species' respective LPS subunits (40, 41). The uniqueness of *Brucella* virulence factors causes changes in the pathogen associated molecular patterns (PAMPs) during infection which tend towards inimitable interactions that are not well characterized (40). *Brucella* LPS contains Lipid A bound to an oligosaccharide core which can be associated, or not, with an antigenic polysaccharide chain called the O-chain or O-chain Antigenic Polysaccharide (OPS). *Brucella* LPSs that contain the O-chain are called smooth-LPS (S-LPS) and those without are called rough-LPS (R-LPS). The overall S-LPS molecule is amphipathic as the Lipid A portion is hydrophobic and the O-chain is hydrophilic. Most *B.*

abortus strains conform to the smooth phenotype (40, 42), however, there are exceptions to this such as the non-virulent *B. abortus* str. RB51, an attenuated isolate used for vaccination as a preventative measure against bovine brucellosis (42). In bovine serodiagnostic testing, most assays are designed to detect antibodies against the *B. abortus* S-LPS, which can indicate exposure to a virulent smooth strain of the bacteria. LPS antigens elicit powerful host immune responses in cattle which is why their use is common (43). However, such tests encounter significant challenges, which will be discussed later.

Wild-type strains of all classical *Brucella* species carry S-LPS except *B. ovis* and *B. canis*, which carry R-LPS (44). A benefit of using S-LPS antigens in serodiagnostics is the intense response they elicit from mammalian immune systems, and serological assays testing against S-LPS have been reported to have 100% sensitivity (45). OIE also reports that S-LPS based serological tests rarely produce false negatives, but that S-LPS specificity is low and can't alone be used to give a definitive diagnosis for brucellosis (5). Other Gram-negative bacterial pathogens have O-chain associated S-LPSs that are structurally similar to *Brucella* spp., such as *Vibrio cholera* O1, *Escherichia coli* O:157, and *Salmonella* group N (O:30) (40); especially S-LPS of *Yersinia enterocolitica* O:9 which has an O-chain that is nearly identical to that of *B. abortus* (46). *Y. enterocolitica* is a common human pathogen in developed and developing countries alike (47). *Y. enterocolitica* is also found in the intestinal flora of domesticated swine, ovine, and bovine populations (48). *E. coli* O:157 is also commonly found in up to 50% of healthy cattle that are carrying and shedding it in their feces at any given time (49). As a result of S-LPS cross-reactivity, infection or exposure to the non-*Brucella* Gram-negative pathogens mentioned above can cause False Positive Serological Results (FPSR) in serodiagnostic testing. This issue is especially troublesome in countries that have achieved or nearly achieved

brucellosis-free status (50). FPSR has been a problem for over 100 years beginning with bacterial cross reactions detected in Serum Agglutination Tests (SAT) (51). Thus, confirmatory assays are needed to follow up on any positive results from S-LPS based testing (5).

The gold standard for brucellosis diagnosis is isolation and recovery of the bacterium from the blood, bone marrow, or tissues of the infected host (52), although cases have been reported of animals yielding negative culture results even though they are indeed brucellosis positive (53). *Brucella* isolation is usually achieved using a selective media; Farrell's formulation is the most common (54). *Brucella* spp. grow slowly with a doubling time of around 3 hours (55). Correspondingly, culturing *Brucella* spp. can be laborious, time consuming and hazardous (19, 52). *Brucella* spp. are easily aerosolized, extremely infectious at low doses and have momentous potential for use in agricultural, civilian, and military bioterrorism (31). *Brucella* cultures must be handled in Level 3 containment facilities with proper biosafety and biosecurity infrastructure in place. Considering the challenging and hazardous aspects of culturing *Brucella* spp*.,* the implementation of culturing methods is impractical in screening for infected animals, which needs to be accessible, rapid, and high-throughput (56). Molecular techniques have been established to improve the ease and accuracy of *Brucella* diagnostics by using the Polymerase Chain Reaction (PCR), in many cases following bacterial enrichment (37). Although genetic loci have been identified to differentiate between *Brucella* species and biovars, PCR sensitivity is often low due to the difficulty of lysing the bacterial cells in host blood samples (57). Additionally, developing countries lack the infrastructure, equipment, and expertise to perform complex molecular work (37). Serological testing could potentially be a solution for eliminating the prolonged growth time and hazards associated with culture-based

brucellosis diagnosis as well as for overcoming the sensitivity issues encountered when using molecular diagnostic methods.

While at this time there are no single serological tests that are sufficient to give a definitive diagnosis of brucellosis (5), work towards developing better diagnostic methods has been ongoing and at the forefront of brucellosis research for the past century (53). According to the 2019 Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by the OIE, brucellosis diagnosis requires positive test results from at least two of culturing, serological, bacteriological, or molecular identification assays (5). This is also the case in humans where serological tests are used for preliminary diagnostics and followed up with culture recovery from blood, bone marrow, or tissue samples from the patient (52).

For bovine brucellosis serodiagnostics, there is a substantially large number of tests that have been developed, many of which have also been modified in several ways over the years to increase performance. Serological tests fall under four broad categories: agglutination assays, complement fixation assays, immunoprecipitation assays, and primary binding assays which include immunosorbent assays and fluorescence assays (44, 51, 53). Currently, there are only a handful of validated standardized assessment techniques recommended for use internationally by the OIE that will be discussed in detail. These are the Complement Fixation Test (CFT), the Fluorescence Polarization Assay (FPA), and Enzyme-Linked Immunosorbent Assays (ELISAs). FPAs and ELISAs are primary binding assays. All of these tests use either S-LPS, OPS, whole smooth-brucellae preparations, or a combination of these as antigens (43, 44). The SAT has been historically important but is less common now as it is outperformed by CFTs, FPAs, and ELISAs. The tests recommended by OIE for local screening for *Brucella* exposure and infection are categorized as Buffered *Brucella* Antigen Tests (BBATs). BBAT format recommendations

are exclusive to the Rose Bengal Test (RBT) and the Buffered Plate Agglutination Test (BPAT). FPAs and ELISAs are suitable for local surveillance as well (5). Tests for local screening are generally designed to be inexpensive, rapid, and highly sensitive, but are not always highly specific (53).

The SAT is described by the OIE as unsatisfactory for modern international cattle trading (5). Brucellosis SATs are still common in clinical settings where multiple serum samples can be evaluated for infection over a period of several weeks (58). Results from brucellosis SATs allow qualitative visualization of antibodies clustering around S-LPS antigens. CFTs outperform SATs and have been used to diagnose humans and bovine brucellosis for nearly a century (59, 60). According to OIE, CFT is a complicated assay to perform and involves tedious maintenance of the required biological reagents. Modern CFT methods consistently outperform SATs in terms of specificity. Further, quantitative units have been standardized for CFTs (5). CFT assays require serum antibodies to bind to the antigen of interest, for added complement proteins to bind to that complex, and for red blood cells (RBCs) to bind to the complement. The presence of RBCs aggregated around complement proteins indicate a positive reaction and can then be quantified. For *B. abortus* CFT serological testing, the antigens employed are usually *Brucella* S-LPSs, as smooth strains are the most commonly isolated from outbreaks (42, 43). The performance of FPA is comparable with CFT, often better (5). FPAs were established through the 1960s and 70s with applications in human medicine (61, 62). Development of FPA for detecting antibodies to *B. abortus* in bovine sera was published by the CFIA in 1996 and had been developed for use on domestic herds (63). FPA involves measuring the spin of fluorescently tagged molecules in liquid media based on the principle that larger molecules spin slower than smaller ones. Antigens are labelled with fluorochrome markers and when antibodies against them are present in the

serum, they bind to the antigens and the newly formed complexes spin more slowly. Sample spin rates are then quantified through comparing spin rates of known positive and negative samples (62). ELISAs are another common serodiagnostic technique and their performance is also reported to be comparable with, or better, than CFT. Two standardized forms of ELISAs used for brucellosis testing are the indirect (I-ELISA) and competitive (C-ELISA) (5). A visualization of the common formats that ELISAs can take is shown in Figure 1. There are many variations on both approaches for antigen preparations, antibody conjugations, and substrates. However, only certain approaches have been validated for international use by the OIE. I-ELISAs are performed with plates coated with the antigen of interest that are exposed to serum antibodies. Conjugated reporter antibodies then bind to antigen-bound serum antibodies and produce a signal, such as a colour change, to indicate a positive result (64). I-ELISAs following OIE guidelines must all be run with positive and negative controls as well as an additional positive serum included in each plate as an internal control. Additionally, only *B. abortus* strain 99, *B. abortus* strain 1119-3 or *B. melitensis* strain 16M can be used for antigen production. *Brucella* antigens for I-ELISAs are recommended to be S-LPS or OPS ideally, but whole cell preparations may also be used as antigens if validated under the recommendations. ELISAs using S-LPS and OPS have high sensitivity comparable to CFT and BBAT but generally perform with slightly lower specificity (5, 65). I-ELISA using S-LPS or OPS are not capable of differentiating vaccinated animals from those infected and face problems of FPSR. Use of cytosolic *Brucella* proteins as antigens in I-ELISAs do not resolve these issues (5). C-ELISAs start with a plate coated with antigens that serum antibodies of interest can bind to. Serum antibodies and conjugated monoclonal antibody (mAb) reporter molecules that compete for binding to the immobilized antigens are then added to the plate wells. Serum samples that bind to the antigen will reduce signal strengths generated

from binding of the competing monoclonal antibody to the antigen. Sample results can then be compared to positive and negative controls (64). C-ELISAs performed under OIE recommendations use S-LPS or OPS antigens with OPS being reported to often have higher specificity. However, C-ELISAs using OPS are still susceptible to FPSR. The increased specificity of C-ELISAs requires sacrification of some sensitivity when compared to I-ELISA and BBATs. Regardless, any positive results must still be subjected to confirmatory testing (5, 43). Like I-ELISAs, C-ELISAs following OIE guidelines must all be run with positive and negative controls as well as an additional positive serum included in each plate as an internal control (5). OIE recommended BBATs are the RBT and the BPAT. RBTs are stained plate agglutination tests. RBTs conforming to the OIE recommendations must use antigens sourced from *B. abortus* S99 or S1119-3. RBTs are susceptible to FPSRs from vaccinated samples and cross-reacting bacteria (5). BPATs are stained agglutination tests performed in well plates. BPATs conforming to the OIE recommendations must use antigens sourced only from *B. abortus* S1119-3, and brilliant green and crystal violet must be used as the stains. BPATs are sensitive to vaccinated samples and false-negatives if antibody concentrations are out of range, which is referred to as prozoning (5).

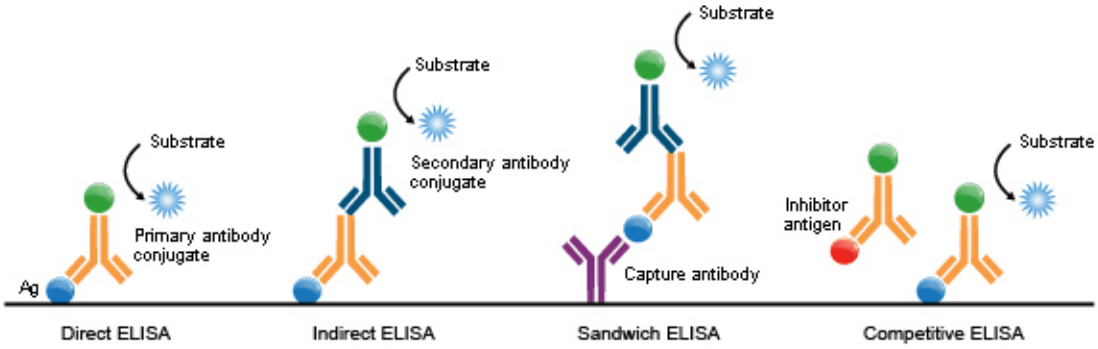


Figure 1: Types of ELISA Methods. Four major ELISA approaches presented. Ag stands for antigen. Primary antibodies are orange, secondary antibodies are blue, and anchored antibodies are purple. Conjugated reporter enzymes are green. Diagram sourced from abnova.com (66).

To overcome the issue of FPSRs, the OIE recommends following up positive serological results with a secondary, confirmatory serological test. Serological positivity may show exposure to LPS but not necessarily *Brucella* LPS as discussed above. Suspect animals are those that test positive to both a screening and confirmatory serological test in series and must then be further confirmed by culture, PCR, and/or the Brucellin Skin Test (BST) in unvaccinated livestock (5, 67). In the European Union (EU), flock screening is generally done with RBTs or I-ELISAs with CFT and C-ELISA used for confirmatory follow ups (50). The OIE recommends that positive serological results should also include epidemiological investigations. The susceptibility of brucellosis diagnostics to FPSR is why multiple rounds of testing are required to confirm *Brucella* exposure and infection. To solve the FPSR problem, it is necessary to develop new diagnostic tools and improve upon the ones already in use (50). Many countries, including Canada, invest great effort and resources into this cause. For example, the Canadian Government's Genomics Research and Development Initiative (GRDI) includes funding for bovine brucellosis diagnostic research. One of GRDI's directives is the application of whole genome sequencing for the high-throughput discovery of novel diagnostic antigens for bovine brucellosis serodiagnostics (68).

Of the established serodiagnostic tests described above, several are also important clinically for the diagnosis of brucellosis in humans. Brucellosis is historically difficult to identify and diagnose clinically due to its variable and often non-specific symptoms (22). Several blood samples are usually taken to test antibody titres over a period of time and are followed up with culturing. Tests to assess human anti-*Brucella* antibody titres include microagglutination, SATs, and Coombes (which is also an agglutination assay) (58). As mentioned, FPA has also been used in human medicine extensively for many years (61, 62) and RBT is appropriate in

clinical settings as well (69). Furthermore, any serological test that performs well in animals can theoretically be used on humans because, in general, there is no difference in testing human and animal serology (51).

Serological testing for antibodies against protein antigens has been proposed as a solution to S-LPS FPSRs and several protein candidates will be discussed later. Currently, there is no reporting on the use of protein-based tests in human or veterinary medicine, however, this may change in the future. The brucellosis research community continues working towards building libraries of *Brucella* protein antigens with the objective of improving the diagnostic specificity and sensitivity of bovine brucellosis serodiagnostics.

II. Immunogenic Protein Antigens and Genomics-Based Discovery

i. Protein Characteristics Conducive to Immunogenicity

There are several key characteristics of bacterial proteins that are hypothesized to enable them to elicit detectable responses from mammalian humoral immune systems: proteins that are secreted and extracellular, proteins that are localized in the cellular envelope (CE), and proteins that contain a signal peptide. Presence of one or multiple of these characteristics is common in previously identified protein antigens that have been described in the literature, as discussed below.

Extracellular proteins secreted by a bacterium likely interact directly with the host immune system (70). In the mammalian immune system, macrophages function to phagocytise particles and cells recognized as foreign, which may include extracellular or secreted protein antigens (71). Following phagocytosis, macrophages present digested antigens to the adaptive immune system which can elicit the humoral immune response (72). The outer membrane, periplasmic space, and cytoplasmic membrane are all part of the *Brucella* CE. Proteins localized

in the CE facilitate bacterium-host interactions and are first to interact with animal hosts (31). Consequently, CE localized proteins have the potential to be immunogenic and have also been reported in some studies (37, 73). Conversely, the presence of signal peptides in bacterial proteins can target them towards secretory pathways (74). Proteins with signal peptides can, thus, confer immunogenicity due to their possible extracellular localization, however, not all secreted extracellular proteins contain signal peptides (75). Additional functions of signal peptides have also been demonstrated. In some cases, signal peptides remain uncleaved in certain classes of bacterial proteins, such as mammalian cell entry proteins (76). Furthermore, many bacterial membrane proteins are anchored into the membrane by uncleaved signal peptides (74). Signal peptides have also been characterized as overall hydrophobic, containing high epitope densities, and adhering to specific molecular templates resulting from amino acid sequence combinations. Mammalian Major Histocompatibility Complexes (MHC) are also reported to be specifically adapted to recognize amino acid arrangements that result in signal peptide formation (77). Signal peptides generally adhere to a universal pattern, however, there is no specifically conserved sequence of amino acids. Signal peptides evolve rapidly and are highly variable throughout eukaryotic and prokaryotic populations, but are universally comprised of three distinct domains: a positively charged amino terminal, a hydrophobic central region and a polar carboxyl terminal (74).

ii. *Brucella* Protein Antigens Discovered to Date

In the scientific literature, *Brucella* proteins have been previously reported to have potential for use as serodiagnostic antigens. Protein antigens discovered to date in various *Brucella* spp. generally have at least one of the characteristics outlined above (78, 79, 80). Current data indicates that protein antigens elicit less intense humoral immune responses than do

LPSs and their subunits (41, 80). Theoretically, this may lead to issues of false negative serological results depending on antibody titers. OIE reports that cytosolic proteins are not specific enough to differentiate between *B. abortus* infection and vaccination in cattle using I-ELISAs (5). However, if newly discovered cytosolic proteins possess potentially immunogenic characteristics, they may still be worth investigating.

Brucella protein antigens have been discovered and published in recent years; several prominent studies are outlined here. Gupta *et al*. in 2010 described the outer membrane protein (OMP) 28 from *B. melitensis* 16M which was identified as an immunodominant antigen recognized by antibodies from sheep, goats, cattle, and humans with confirmed brucellosis. Recombinant OMP28 from *B. melitensis* was then explored in the development of an ELISA screening test (78). Additionally, Lim *et al*. in 2011 evaluated OMP28 from *B. abortus* 544 as an antigen for use in clinical bovine brucellosis diagnostics through western blot assays and ELISAs. They show sensitivity, specificity, and accuracy of screening tests using OMP28 all above 95% (79). A proteomic study by Kim *et al.* identified 11 protein antigens with potential for use in serodiagnostics. In that study *B. abortus* RB51, a mutant strain lacking LPSs, was used as the protein source. Proteins were separated by electrophoresis and probed with 4 types of sera – *B. abortus* positive*, Y. enterocolitica* O:9 positive, *E. coli* O157:H7 positive, and *B. abortus* negative – in Western blotting to characterize immunogenicity and cross reactivity. The study demonstrated that all 11 of the proteins identified have high serodiagnostic screening potential with considerably reduced cross reactivity (80). Moreover, Rossetti *et al*. have demonstrated a 26 kDa periplasmic protein from *B. abortus* S19 capable of differentiating infected bovines from those vaccinated, and of identifying infected rams through serum screening with western blots. They assert this protein antigen has worth in the diagnosis of other mammals exposed to

Brucella as well (73). Additionally, Yin *et al.* in 2016 reported a synthesized immunoreactive recombinant protein candidate for diagnosis of human brucellosis by ELISA, referred to as rOMP in the study. In the study, they also discuss the previously identified *Brucella* spp. proteins OMP16, OMP25, OMP2b, OMP31, and periplasmic protein 26 (BP26) that they used as reference antigens in their experiments and protein design (37). Yin *et al.* also note that testing in more strains is needed to confirm their work. Large scale serum testing would also allow for determination of the statistical significance of serological results using protein antigens.

At this point in time, there are no reports of discovered protein antigens from the literature being validated and standardized for serodiagnostic use by an international regulatory body such as the OIE. Correspondingly, no protein-based serological testing approaches are currently recommended (5). While specific issues associated with serological testing using previously identified protein antigens have not necessarily been identified as of yet, large scale validation is needed before such diagnostic assays can be integrated into international testing regimes. Furthermore, a new test, once validated, would need to be equal to or superior in diagnostic performance to existing methods. It would also need to be cost effective in order to be a replacement for any standardized methods currently recommended (53).

The discovery of non-LPS *Brucella* antigens in the literature justifies further research seeking to identify novel *B. abortus* immunogenic proteins and continuing the work of building antigen libraries and setting scientific foundations for future applications. Additionally, research needs to be performed to address the gap in the literature for the reference strain 86/8/59 of *B. abortus* bv. 2, where little research has yet been done but which is one of the most frequent biovars to be isolated from bovines and humans (33, 34).

iii. *B. abortus* bv. 2 str. 86/8/59 Genomic Data

At the time of writing, there are two key annotated whole genome sequences (WGS) of high-quality on NCBI GenBank's Non-Redundant Nucleotide (nr/nt) database for *B. abortus* bv. 2 str. 86/8/59 specifically. Both of these *B. abortus* sequences are identical, however, one is annotated under NCBI's RefSeq designation as a reference sequence for *B. abortus* while the other retains the original annotations. The RefSeq annotated genome file accession numbers on GenBank are NZ_CP007765.1 and NZ_CP007764.1 for chromosome 1 and 2, respectively, and the genome accession numbers annotated under the original format are CP007765.1 and CP007764.1 for chromosome 1 and 2, respectively. Separate nucleotide alignments using NCBI's Basic Local Alignment Search Tool (BLAST) of chromosome 1 accessions NZ_CP007765.1 and CP007765.1 together and chromosome 2 accessions NZ_CP007764.1 and CP007764.1 together both show 100% identity between the query and subject with Expect (E) values of 0.0. E values of 0.0 indicate that the alignments are statistically significant and are not expected to have occurred by chance (81). NZ accession number designations on RefSeq files can specify either complete genomes or unfinished WGS data (82). In the cases of the genome accession numbers outlined above, all are listed as complete on their respective GenBank webpages. NCBI's RefSeq is a non-redundant (nr) database of reference standard sequences of complete genomes, chromosomes, organelles, viruses, plasmids, intermediate assembled genomic contigs, curated genomic regions, mRNAs, RNAs, and proteins. RefSeq is an international effort that includes contributions from North America, Europe, and Japan (83). Accession number labelling of individual protein coding genes within those two *B. abortus* bv. 2 str. 86/8/59 genomes vary as a result of the differing aforementioned annotation formats. However, a common labelling system is present for gene locus tags across both WGS files,

allowing for cross-referencing and the avoidance of redundancy. Protein coding gene accession numbers in the non-NZ designated chromosomes are labeled with AIJ prefixes, and locus tags are labelled with DK55 prefixes. Protein sequences in the RefSeq chromosome files with NZ accession numbers are labelled with WP prefixes, and locus tags are labelled with DK55 prefixes. Reference protein coding gene sequence locus tags are also labelled under a second, separate designation system using DK55_RS prefixes (84). WP accession numbers are used for reference protein curation to ensure non-redundancy of homologous sequences across multiple strains and species in the nr database (82). WP prefixed accession numbers and NCBI's nr database are discussed in more detail later on in the Strategies for Homologous Protein Identification section.

iv. Machine Learning and Antigen Discovery

A popular bioinformatics method for analysis of bacterial protein sequences to determine subcellular localization is PSORTb. PSORTb employs BLAST database homology analysis and alignment algorithms, HMMTOP transmembrane helix projection algorithms, and motif and profile matching modules, all alongside support vector machine learning, to make bacterial protein subcellular localization predictions based on protein sequences specified by corresponding ORFs. Final localization predictions are generated by a Bayesian network that relies on the outputs of the analytical modules listed above (85). PSORTb has been shown to accurately predict protein locations with the lowest percentage of false positives out of all protein location prediction software available (85) and is the most precise bacterial localization prediction tool available (86, 87, 88). PSORTb also functions to detect signal peptides in protein amino acid sequences through analysis by Markov chain algorithms from an integrated tool called SignalP (88, 89).

PSORTb whole genome analyses could also have applications in identification of potentially immunogenic protein antigens. This can be done by selecting candidates from the results that have immunogenically favourable localizations and characteristics, such as being in the cell envelope or membrane and having signal peptides. Use of PSORTb in this way illustrates a multidisciplinary approach by merging the fields of immunoproteomics and genomics. Such a merger opens up bacterial genomes to the high-throughput discovery of protein antigen candidates, as there are many uncharacterized proteins encoded by ORFs available that can be evaluated for immunogenicity.

Other bioinformatic approaches and platforms implementing machine learning algorithms, and specific proteomic and immunoproteomic training sets for those algorithms, have been reported for predicting protein characteristics from sequencing data of uncharacterized candidates. In 2006, Gardy and Brinkman discussed Proteome Analyst, SubLoc, CELLO, PSLpred, LOCtree, and P-CLASSIFIER for protein subcellular localization. Proteome Analyst uses sequence annotation keywords as well as BLAST alignments against the SwissProt database; SubLoc performs amino acid composition analyses of proteins; CELLO analyzes amino acid composition, sequence order, and associated physiochemical properties; PSLpred is similar to CELLO except it analyses dipeptide composition alongside physiochemical groupings; LOCtree uses a flowchart type decision making process designed to copy cellular sorting; and P-CLASSIFIER processes sequencing data by creating fragments of one to four amino acids in length to predict the localization of the query subject by examining their physiochemical properties against known grouping standards. Gardy and Brinkman also overview several programs that perform localization and sequence related predictions of protein properties that operate in a similar way to SignalP. Phobius, for example, predicts the presence of

transmembrane α-helices and signal peptides of query subjects against previously characterized proteins. Other programs are described such as TatP which predicts the presence of TAT-transporter signal peptides. Further, BOMP, Prof-TMB, and TMB-Hunt all analyze amino acid sequences to predict for the presence of β-barrels in OMPs (85). In 2010, Yu *et al.* outlined PSL101, PSLDoc, Gpos-PLic, Gneg-Ploc, SubcellPredict, HensBC, LocateP, Auger, and TBPred in a comparison of protein localization prediction strategies. PSL101 predicts localization from amino acid compositions and conserved structural protein features and PSLDoc performs gapped dipeptide analyses. Gpos-PLoc and Gneg-PLoc cluster the query subject with SwissProt curated protein sequence localization annotations of statistically similar amino acid properties to make predictions of localization based on the matches. SubcellPredict and HensBC implement multiple algorithm combinations to boost prediction and classification performance. LocateP and Auger are designed specifically for Gram-positive bacteria and differentiate between anchored and secreted proteins. TBPred was designed specifically for *Mycobacterium* spp. which aimed to increase prediction ability by using genus specific training data for the machine learning algorithms (88). More recently, Yin *et al.* in 2016 used three software programs – Bepipred, ABCpred, and COBEpro – that predict B-cell epitopes from protein sequences of known immunoreactive proteins. This allowed them to synthesize immunogenic candidates relevant in human brucellosis diagnostics. Additionally, following bioinformatic analysis by these programs, they determined candidate homology between *Brucella* spp. using NCBI's BLASTP (37).

v. Bioinformatic Strategies for Homologous Protein Identification

Tools are available on NCBI that can help determine organismal specificity of protein candidates once they have been identified. Amino acid sequences can be analyzed on NCBI's

interface using the Identical Protein Groups (IPG) search function. IPG scans the NCBI Non-Redundant (nr) protein database for the presence of the same protein in other submitted genomes. Database query outputs can be sorted to limit result reporting to include or exclude specific taxonomic lineages (90, 91). IPG searches include access to GenBank, RefSeq, and SwissProt databases. Lowest common taxonomic group names are indicated where the identical protein has been observed multiple times, and the number of identical proteins in each group can be used to identify those that are highly conserved. RefSeq proteins using the non-redundant "WP" accessions may be annotated on many distinct genomes (90). When the NCBI genome annotation pipeline annotates a bacterial protein that is 100% identical and the same length as an existing non-redundant protein on RefSeq, that protein is given the same "WP" accession number in the annotated coding sequence (CDS) feature. Results indicate that the protein is found in more than one genome that share a common taxonomic node, and non-redundant protein records always represent one exact sequence that has been observed once or many times in different strains or species (91). Once a protein candidate sequence has been demonstrated to be unique, more information can be gathered, such as protein evolution, from database calling of similar, but not identical, sequences in other genomes and bacterial lineages. Degrees of homology to other proteins can then be determined using bioinformatic methods such as NCBI's BLASTP for each protein candidate.

III. Strategies for Protein Antigen Discovery in *Brucella*

Protein candidates, once prepared, can be evaluated for their immunogenicity. Several ways of accomplishing this are outlined, specifically for *Brucella* spp., in the studies described previously that reported on proteins found to be immunoreactive when tested against brucellosis positive serum. Rossetti *et al.* identified the *B. abortus* periplasmic protein BP26 in 1996 and

used conventional western immune blotting with sera from naturally infected, vaccinated, and healthy cattle (73). In 2010, Gupta *et al.* described an outer membrane protein from *B. melitensis*, also termed BP26. Immunogenicity was determined by complement fixation, SAT, and ELISA assays with serum sourced from vaccinated and naturally infected animals (73). Lim *et al.* in 2011 tested an outer membrane protein from *B. abortus* produced through recombination called rOMP28. Through western immunoblotting, agglutination assays and ELISAs they determined rOMP28 was immunoreactive with serum sourced from Korean cattle herds (79). In 2014 Kim *et al.* identified 11 immunoreactive candidates through western immunoblots with serum sourced from artificially inoculated cattle. Cattle serum infected with commonly cross-reacting pathogens was also used as negative controls (80). Additionally, in 2016 Yin *et al.* reported a protein antigen with potential applications in human brucellosis serodiagnostics. The recombinant outer membrane protein, called rOMP in the study, was created synthetically through integration of bioinformatics techniques analyzing B-cell epitopes of known immunogenic OMPs across several species of *Brucella.* The study evaluated immunogenicity of the protein through I-ELISAs and western blotting (37). These studies all profiled humoral immune responses to specific protein candidates in similar ways for the discovery of brucellosis diagnostic antigens. Detecting specific antibodies in positive serum samples is the core principle of the work in these studies. Furthermore, Davies *et al.* in 2005 did research centered around identification of protein antigens with applications for vaccine development for vaccinia virus (92). Methods used in that study have many parallels with the strategies implemented in this thesis for immunogenicity evaluation, which are described later.

1.3 Hypothesis

Development of a selection and screening workflow for high quality profiling of bovine humoral immune responses to *Brucella abortus* bv. 2 str. 86/8/59 proteins will contribute to the discovery of novel immunogenic antigens capable of improving bovine brucellosis serodiagnostic specificity compared to current LPS-based methods.

1.4 Objectives

1. Bioinformatics-based discovery of *B. abortus* bv. 2 str. 86/8/59 open reading frames coding for extracellular, cell envelope localized, or signal peptide-containing proteins, unique to the genus, through analysis of the bacterial genome and NCBI protein database.

2. Molecular cloning, *in vitro* expression, and purification of selected protein candidates for downstream immunological reactivity evaluation.

3. Evaluation of reactivity of successfully expressed protein candidates against brucellosis positive and negative serum panels using protein arrays.

2. Materials and Methods

An overview of the experimental workflow and investigational methods implemented throughout the project is shown in the flow chart (Figure 2). The methods presented in this section are a combination of those outlined in the scientific literature related to the fields of bioinformatics, molecular biology, recombinant protein expression, proteomic processing, and serological evaluation. Also, many of the research strategies applied were standardized protocols within the laboratory group. Furthermore, several of the experimental operations and techniques described were adapted or developed specifically for this project during the research phase. Execution of the research strategies outlined below generated large amounts of data – including bioinformatic and sequencing information. Corresponding in-depth analyses were a significant component of the scientific methods executed in this project and were essential for the acquisition of refined results presented in this thesis.

Figure 2: Flow chart of Experimental Methods. The chart illustrates details of each research phase. Processes are shown in order for the selection, production, and evaluation of *B. abortus* bv. 2 str. 86/8/59 protein candidates.

2.1 Candidate Selection, Production, and Evaluation

2.1.1 Protein Candidate Selection

Genomic reference sequences were downloaded from NCBI RefSeq (ncbi.nlm.nih.gov/refseq) for *B. abortus* bv. 2 str. 86/8/59 in FASTA format (Chromosome 1 reference sequence accession number: NZ_CP007765.1; Chromosome 2 reference sequence accession number: NZ_CP007764.1). Genome files were analyzed in the PSORTb (psort.org/psortb) bioinformatics platform for categorizing the proteins encoded by the genome based on their predicted cellular localization. Protein Candidates were selected from the PSORTb output according to their predicted cellular localization from the Bayesian Network module. This included extracellular or CE proteins, proteins with gene sizes of >0.4 kb and <3 kb, and proteins with signal peptides detected by the SingalP module were also selected. Protein candidates with such properties have the potential to be immunogenic. Additional information on selected protein candidates was obtained through querying candidate accession numbers on NCBI GenBank (ncbi.nlm.nih.gov/genbank). This included gene length in base pairs (bp), protein length in amino acids (aa), and cross-referencing gene locus tags and protein accession numbers. Amino acid sequences of selected candidates were analyzed using NCBI's IPG search function (ncbi.nlm.nih.gov/ipg), which scans the NCBI Non-Redundant (nr) Protein database (ncbi.nlm.nih.gov/refseq/about/nonredundantproteins) for the presence of the same protein in other submitted genomes. Database query outputs were sorted to exclude *Brucella* species and to only display genomes from other genera that contained the queried proteins. Proteins with no identical matches or no similarity to those in genera other than *Brucella* were confirmed for downstream evaluation.

2.1.2 Oligonucleotide Design

Annotated genomic reference sequences were downloaded from NCBI GenBank in standard .gb format and loaded into the SeqBuilder application of the DNASTAR Lasergene ™ (dnastar.com) software package. Candidate genes were selected by searching the genome file for their locus tags. The oligonucleotide design function was utilized to design PCR primers ranging around 15 – 30 bps in length and corresponding to the terminal regions of each candidate gene's ORF. The 5' ends of PCR primer oligonucleotides include target sequences of the pIVEX2.3d cloning vector for generation of PCR ORF amplicons for downstream *in vivo* cloning by homologous recombination. Primers were designed to begin with 5'GTTTAACTTTAAGAAGGAGATATACC3' and 5' GATGATGATGATGATGAGAACCCCC3' for forward and reverse primers, respectively. Forward primers additionally contained a standard ATG start codon if not naturally present. Further, in the portion of the reverse primer sequences corresponding to the C-terminal regions of target genes, TAG, TAA, and TGA stop codon sequences were removed to facilitate expression of recombinant candidate proteins containing C-terminal 6x His-tag coding sequences. Following completion of the oligonucleotide designs, oligonucleotide primers were synthesized by Millipore Sigma (sigmaaldrich.com).

2.1.3 Molecular Approaches to Protein Expression

PCR is a molecular method for various applications including mutagenesis, gene expression, gene cloning, and genome analysis (93). Molecular cloning often relies upon PCR and is also a diverse and essential technique for the fields of genomic study. *In vivo* cloning by homologous recombination, and specifically, utilization of transformation in $CaCl_2$ competent *E. coli,* is a highly efficient method that has come to prominence in recent years. Cloning by transformation in *E. coli* exploits the intrinsic DNA gap repair mechanism of intragenomic recombination, which is the natural process for restoring collapsed replication forks (94, 95). *E. coli* are equipped to assemble circularized recombinant plasmids from PCR amplicons and linearized vectors, which possess overlapping terminal regions (95). A visualization of this process is presented in Figure 3. A popular strain of *E. coli* for this particular purpose is DH5α. It has been demonstrated to have one of the highest efficiencies in the ability of uptaking and recombining DNA fragments by transformation mechanisms under controlled conditions once made competent (95, 96, 97). *E. coli* cells made to be chemically competent have undergone treatment with calcium chloride and heat shock to enable attachment and entry of foreign DNA particles (98, 99). The techniques of PCR and cloning are often used in conjunction when *in vitro* expression of protein is necessary (92). A visual overview of *in vitro* expression is presented in Figure 4. High-quality PCR amplicon samples of target genes and cloning vectors are required. To achieve this, a DNA purity reading of ~1.8 at the A260 nm/A280 nm ratio following purification is optimal (100, 101). High quality recombinant protein candidates are also needed downstream for immunogenicity screening against bovine serum. Ni-NTA chromatography assays can achieve this following *in vitro* expression, this process can be visualized in Figure 5.

pIVEX cloning vectors are circular plasmids with sequences conducive to both *in vivo* and *in vitro* transcription and translation of cloned target genes (102, 103). pIVEX vectors are also compatible with DH5α *E. coli* and confer antibiotic resistance in their natural or recombinant circularized forms allowing for selective propagation and culturing. pIVEX2.3d, specifically, is 3.56 kb and has a multiple cloning site (MCS) bordered with many types of restriction enzyme sequences (104) as can be seen in Figure 6. Histidine (His)-tag coding sequences are present on the pIVEX2.3d vector in the C-terminal region bordering the MCS, also shown in Figure 6. The reverse complement of the C-terminal His-tag region must be included in reverse direction oligonucleotides for its integration into candidate ORFs amplified from genomic extracts. Its integration is also essential for successful His-tagging of expressed candidates to occur during homologous recombination (104). The presence of His-tags on expressed recombinant proteins allows for their detection in immunoblotting as well as their purification through Immobilized Metal Affinity Chromatography (IMAC). His-tags are low in molecular weight and their presence in recombinant proteins does not affect structure or function (105). In addition to the His-tag coding sequence, pIVEX vectors also possess T7 specific promoter and terminator transcriptional sites (103). T7 transcription is specific to the RNA polymerase of the T7 bacteriophage. T7 RNA polymerase can produce large amounts of specific RNA transcripts *in vivo* and *in vitro* (106). Correspondingly, T7 RNA polymerases are often utilized in cell free expression reaction mixtures (104). The activity of T7 RNA polymerase is much higher than the endogenous *E. coli* transcription machinery and has a lower frequency of termination in the absence of T7 specific terminator sequences (107). T7 promoters and terminators are highly uncommon in DNA unrelated to T7, thus, the use of T7 sites is appropriate for complete transcription of almost any target DNA. Additionally, oligonucleotides

can be designed to directly bind to T7 transcriptional sites due to their conserved specific sequences and lengths sufficient for such purposes (106). Oligonucleotides complementary to T7 sites have applications in PCR, including recombinant colony screening, and Sanger sequencing.

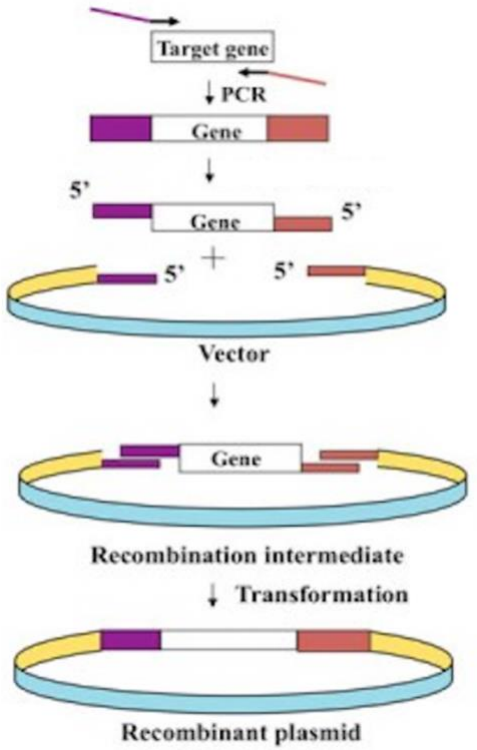


Figure 3: Cloning by Homologous Recombination. Cloning by transformation process in *E. coli* displayed. PCR primers binding to the target gene are represented by the red and purple tailed arrows. Identical sequence regions on the amplified target gene and the linearized vector are shown by matched colours. Overlap of colour matches indicates cloning of the target gene into the vector. Diagram adapted from harvard.edu (108).

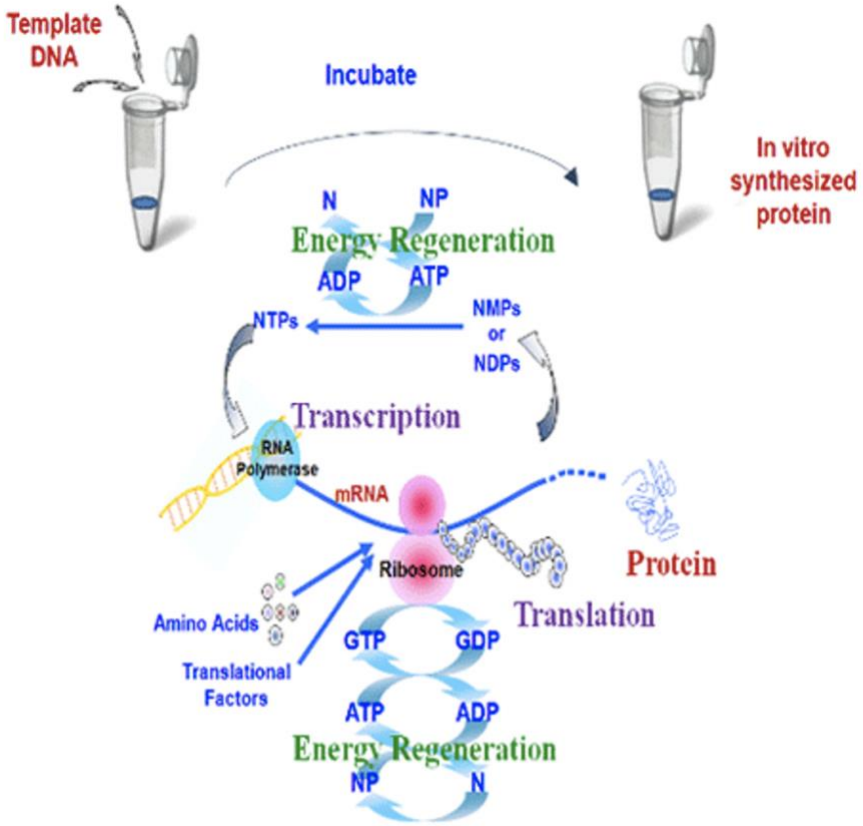


Figure 4: *In Vitro* Transcription and Translation. Cell free *E. coli* lysate-based expression process displayed. Template DNA is incubated in a test tube and the biological machinery is able to perform in the absence of a host when all biomolecules and substrates required are present. Diagram adapted from bioneer.com (110).

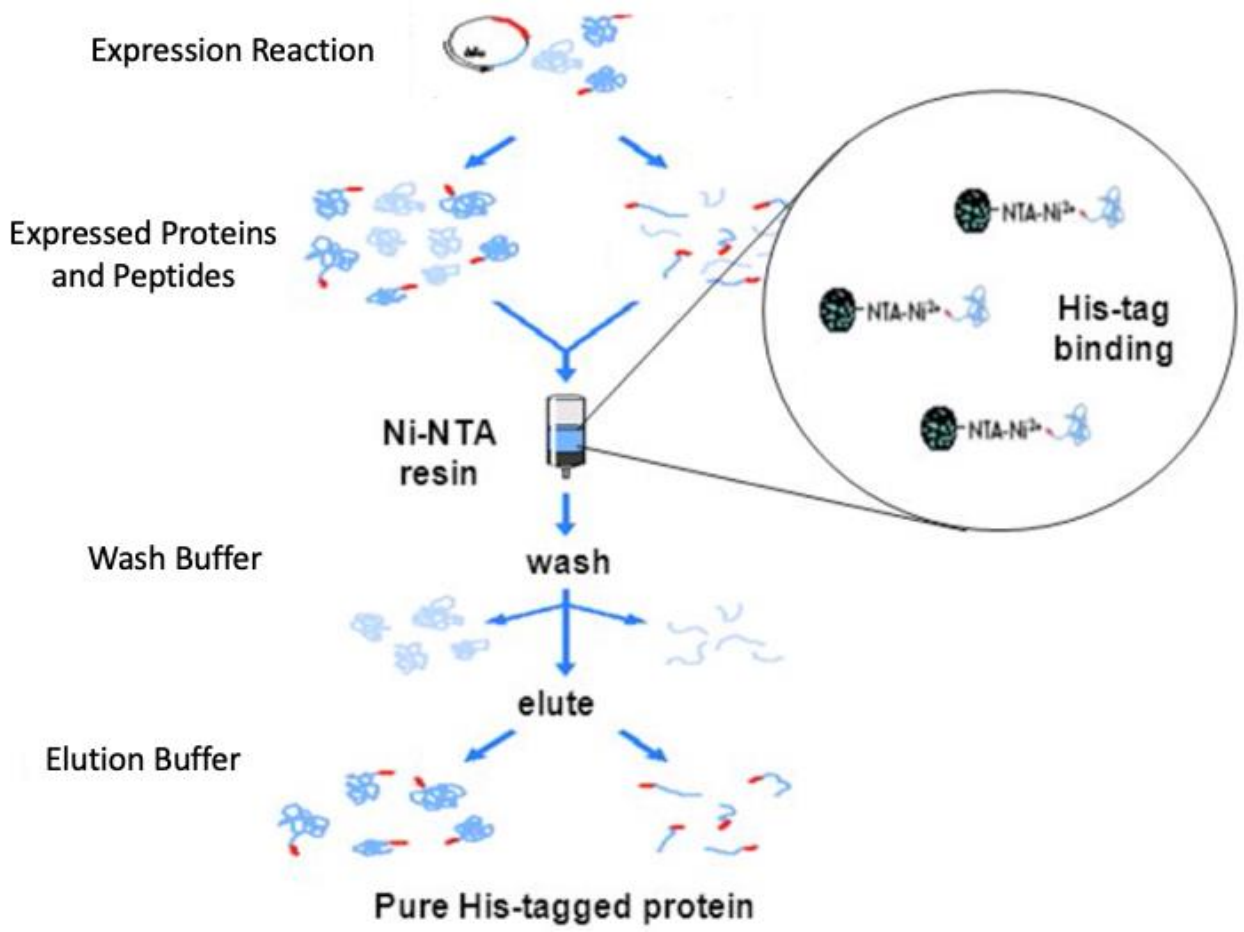


Figure 5: Protein Purification using Ni-NTA His-tag Affinity Binding. Chromatographic purification of proteins and peptides containing His-tags through metal chelate residue binding. Multiple stages of wash cycles remove impurities followed by elution of His-tagged proteins and peptides. Diagram adapted from from Suriyamongkol & Wishart 2006 (111).

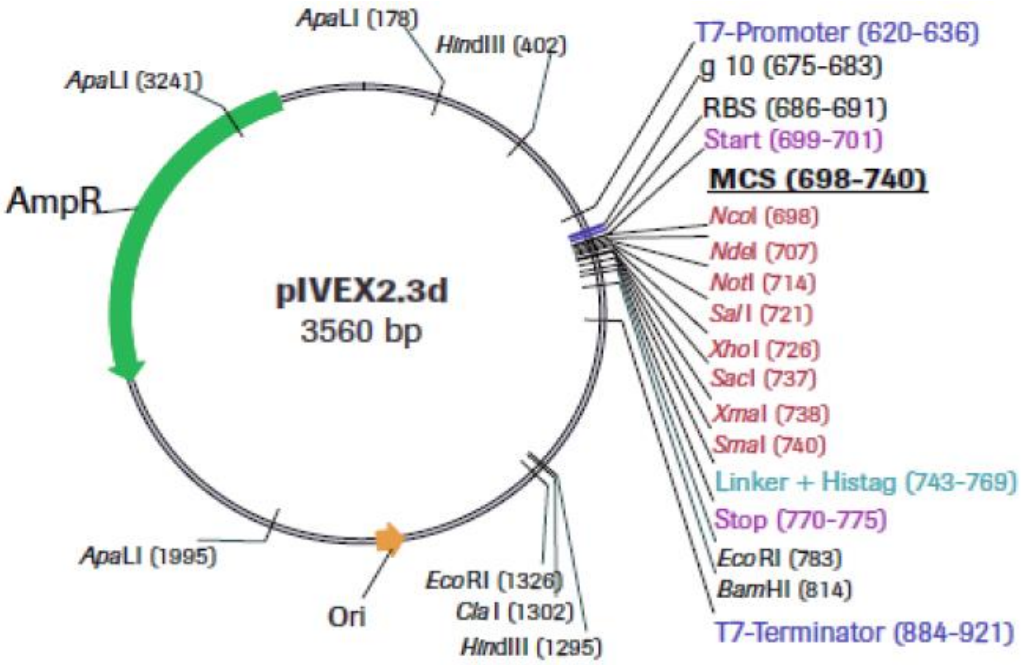


Figure 6: The pIVEX2.3d Cloning Vector. Genetic layout and plasmid length of pIVEX2.3d presented. Positions of the Start and Stop codons, the His-tag sequence, numerous restriction enzyme cut sites, the MCS, the T7 Promotor and Terminator transcription sites, the replication origin (Ori), and the ampicillin resistance gene (AmpR) downstream of the Ori are shown. Diagram adapted from biotechrabbit.com (109).

2.1.4 Protein Production

I. Amplification of ORFs from *B. abortus* Genome

i) Preparation of Template DNA

Heat-killed *B. abortus* str. 86/8/59 glycerol stock samples were resuspended in 100 µL aliquots of sMQH$_2$O using an inoculating loop and centrifuged at 13,000 ^{x} g for 2 minutes. Pellets were resuspended in 100 µL of 50 mM NaOH (0.2 g NaOH (Sigma, USA); 100 mL sMQH$_2$O) and cell suspensions were mixed by vortex for 1 minute and heated at 100°C for 10 minutes. 10 µL of 1 M Tris-HCl (pH 8.0) (recipe A2.1) was added to the samples before additional mixing and centrifugation at 13,000 ^{x} g for 1 minute. Supernatants were stored at –20°C until use in PCR.

ii) Phusion High-Fidelity DNA Polymerase PCR

Resuspension and Dilution of Primers. Lyophilized primers were spun down for 30 seconds at 16,000 ^{x} g and dissolved in sMQH2O to prepare 100 µM stock solutions as per the manufacturer's instructions. Working primer solutions of 25 µM were diluted from stock solutions in sMQH$_2$O.

PCR Reactions. PCR reaction mixtures were prepared as follows: 10 µL of 5X Phusion HF Buffer; 1 µL of 10 mM dNTPs (recipe A2.2); 1 µL of 25 µM forward primer; 1 µL of 25 µM reverse primer; 1 µL of Template DNA (50 – 250 ng/µL); 0.75 µL of Phusion DNA polymerase; 1.5 µL of DMSO; 33.75 µL of sMQH$_2$O.

Thermocycling Conditions. PCR was performed with the following parameters: initial denaturation at 98°C for 30 seconds; 30 cycles of denaturation, annealing, and extension at 98°C for 10 seconds, 56°C for 30 seconds, and 72°C for 30 seconds per kb of target DNA, respectively; final extension at 72°C for 10 minutes; hold at 4°C.

Electrophoresis. Agarose gel (1% w/v) solutions (recipe A2.3) were prepared. Gel solutions were heated by microwave for ~1 minute until the agarose gel powder dissolved completely. Three μL of SYBR Safe (10 mg/mL) was added to the solution before being poured into casting frames with up to two rows of 4 – 15 wells and were allowed to solidify. DNA samples were mixed with 6X loading buffer (recipe A2.4) and loaded into the wells. DNA standards were loaded into separate wells and electrophoresis was run at 120 V for 20 minutes. PCR gel images were taken on a BioRad Gel Documentation System with UV light.

iii) PCR Product Purification

A Qiagen PCR Purification Kit (Qiagen, Germany) was used as indicated by the manufacturer. Five volumes of Buffer PB were added to 1 volume of PCR reaction and mixed. In cases where the solution was orange or violet, 3M sodium acetate (pH 5.0) was added in 10 μL volumes incrementally and mixed after each addition until the solution turned yellow, indicating correct pH. The solutions were applied to spin columns in 800 μL increments, centrifuged at 16,000 ^{x} g for 1 minute, and the flow through discarded. Wash Buffer PE (700 μL) was applied to each column, centrifuged at 16,000 ^{x} g for 1 minute, and the flow through was discarded. The columns were spun for an additional minute at 16,000 ^{x} g and placed in a clean 1.5 mL tube and 50 μL of Buffer EB was added to the center of the column and left for a minimum of 1 minute before the samples were spun at 16,000 ^{x} g for 1 minute and the flow through was collected. Concentrations of purified DNA samples were measured on Implen Nanophotometer P330 and purity was determined according to A260/A280 nm wavelength ratios. Purified PCR products were stored at –20°C until use.

II. Recombinant Plasmid Isolation

i) Media Preparation

Luria-Bertani (LB) Broth. Sterile LB Broth (Becton Dickinson (BD), USA) (recipe A2.5) was prepared according to the manufacturer's instructions. Solutions were heated with frequent agitation to completely dissolve the powder and autoclaved at 121°C for 15 minutes. Sterile broth was covered and stored at room temperature (RT) until use.

LB Agar. Sterile LB Agar (BD, USA) (recipe A2.6) was prepared according to the manufacturer's instructions. Agar solutions were heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Solutions were autoclaved at 121°C for 15 minutes, covered, and stored at RT until use.

SOC Media. SOC media solutions (recipe A2.7) were sterilized by autoclave at 121°C for 15 minutes and cooled down to 60°C before the addition of 2 mL 1 M glucose (recipe A2.8). Solutions were stored at –20°C until use.

ii) Vector Preparation

Plasmid Isolation. pIVEX2.3d cloning vector DNA samples were isolated from *E. coli* cultures using a Qiagen Minipreparation Kit (Qiagen, Germany) according to the manufacturer's instructions. *E. coli* cells containing pIVEX2.3d (biotechrabbit, Germany) were streaked on plates of LB agar containing 100 μg/ml carbenicillin (recipe A2.9) and incubated at 37°C overnight. Five mL of LB broth containing 100 μg/ml carbenicillin were spiked with a single colony from the *E. coli* streak plate and incubated at 37°C overnight. Broth cultures were centrifuged at 16,000 ^{x} g for 3 minutes and the supernatant discarded. Cells were resuspended in 500 μL of Buffer P1 (Qiagen, Germany). Buffer P2 (500 μL) was added and the tubes were gently inverted 4 – 6 times without vortex mixing. Buffer N3 (700 μL) was added and the tubes were inverted gently and immediately 4 – 6 times without vortex mixing. Samples were centrifuged at 16,000 ^{x} g for 10 minutes at 4°C and the supernatants were added to spin columns

by pipetting in 800 μL increments. The columns were centrifuged at 16,000 ^{x} g for 1 minute and the flow through was discarded. Wash Buffer PB (500 μL) was added to each column and centrifuged at 16,000 ^{x} g for 1 minute and the flow through discarded. Wash Buffer PE (750 μL) was added to each column before centrifugation at 16,000 ^{x} g for 1 minute and discarding the flow through. Columns were centrifuged at 16,000 ^{x} g for an additional minute and the flow through was discarded. Spin columns were placed in clean 1.5 mL tubes and 50 μL of elution Buffer EB was added to each column and left for a minimum of 1 minute before the samples were spun at 16,000 ^{x} g for 1 minute. The flow through was saved and stored at –20°C until use.

Digestion of Plasmid Vectors. pIVEX2.3d plasmid vectors were extracted from 5 mL of pIVEX2.3d *E. coli* broth cultures (LB broth with 100 μg/ml carbenicillin) using the Qiagen Minipreparation protocol described previously. Digestions leading to vector linearization were performed with NdeI enzymes (NEB, USA). Digestion reaction were composed as follows: 1 μg purified pIVEX2.3d DNA; 5 μL 10x CutSmart Buffer; 1 μL of NdeI enzymes (concentration 10 U/μL) (NEB, USA); sMQH_2O up to 50 μL. Reaction mixtures were spun briefly and incubated at 37°C for 1 hour and 65°C for 20 minutes in an Eppendorf Mastercycler (Eppendorf, Germany). Linearized plasmid samples were stored at –20°C until use.

Purification of Digested DNA Fragments. Digested plasmid purification was performed using a Qiagen Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Desired DNA fragments were excised from agarose gel under a UV lamp using a scalpel following electrophoresis in 1% agarose at 120 V for 20 minutes as previously described. The gel slices were weighed in 2 mL tubes with a maximum weight of 400 mg. Three volumes of Qiagen Buffer QG were added to 1 volume of gel where 100 mg of gel is equivalent to 100 μL of buffer. Samples were incubated at 50°C for 10 minutes with vortex mixing every 2

minutes. Once the gel had dissolved completely, if the solution was orange or violet, 3M sodium acetate pH 5.0 was added in 10 μL volumes incrementally and mixed after each addition until solutions became yellow. One volume of 70% isopropanol was then added into the solution. Samples were applied to QIAquick spin columns in 800 μL increments and centrifuged at 16,000 ^{x} g for 1 minute and the flow through was discarded. Buffer QG (500 μL) was added to the spin columns and centrifuged at 16,000 ^{x} g for 1 minute and the flow through discarded. Buffer PE (750 μL) was added to the columns and centrifuged at 16,000 ^{x} g for 1 minute and the flow through was discarded. The columns were spun for an additional minute at 16,000 ^{x} g and placed in a clean 1.5 mL tube. Buffer EB (50 μL) was added to the center of each column and left for a minimum of 1 minute before the samples were spun at 16,000 ^{x} g for 1 minute. The flow through was saved and stored at –20°C until use.

PCR Amplification of Linearized Plasmid Vectors. Lyophilized primers were spun at 16,000 ^{x} g for 1 minute, dissolved in sMQH$_2$O to obtain a 100 μM stock solution as per the manufacturer's instructions, and diluted to working concentrations of 25 μM. Linearized pIVEX2.3d was amplified by PCR as follows. Forward primer LIN1412 sequence: 5'GGGGGTTCTCATCATCATCAT3'; reverse primer LIN1413 sequence: 5'GGTATATCTCCTTCTTAAAGTTAAAC3'. Phusion PCR reaction mixtures and conditions were prepared as described previously. Amplified pIVEX2.3d DNA samples were purified using the Qiagen PCR purification protocol described previously. Concentrations of purified DNA samples were measured on Implen Nanophotometer P330 and purity was determined according to A260/A280 nm wavelength ratios. Purified PCR products were stored at –20°C until use.

iii) Competent Cell Preparation

E. coli DH5α cells were streaked on a plate of LB agar and incubated at 37°C overnight. LB broth (5.0 mL) was then spiked with one colony from colonized LB agar and cultured overnight at 37°C at 200 rpm. Overnight Broth cultures were sub-cultured at 1/100 dilution in 40.0 mL of LB broth at 37°C and 225 rpm until OD 600 nm reached ~0.4 to achieve a viable cell concentration no greater than 10^8 cells/mL. The culture was then transferred to sterile ice-cold 50 mL polypropylene tubes and cooled on ice for an additional 10 minutes. The culture tubes were spun at 2,700 ^{x} g at 4°C for 10 minutes and the supernatant was discarded by decanting. The pellets were resuspended in 24 mL of ice-cold 80mM $MgCl_2$ – 20 mM $CaCl_2$ solution (recipe A2.10) and rotated on ice on an IBI Scientific Belly Dancer shaker for a minimum of 20 minutes to fully resuspend the pellets. The culture tubes were then spun at 2,700 ^{x} g at 4°C for 10 minutes before the supernatant was discarded by decanting. Pellets were resuspended in 1.6 mL of ice-cold 0.1 M $CaCl_2$ in 10% glycerol (recipe A2.11) per 40.0 mL of original culture and rotated on ice on the Belly Dancer until the pellets were fully resuspended. Aliquots (40.0 μL) of bacterial suspensions were then dispensed into cryotubes and chilled at –20°C before being transferred to –80°C storage until use.

iv) Cloning and Transformation

Preparation of Vector/Insert Mixtures. For preparation of a mixture of vector and insert DNA mixture, 2:1 ratios (w/w) of target ORF DNA (insert) to linearized vector were used. A minimum of 200 ng of target DNA samples were combined with a minimum of 100 ng of purified linear pIVEX2.3d vectors for use in downstream *in vivo* recombination. No samples were added in a volume less than a 1 μL.

Transformation of $CaCl_2$ Competent *E. coli.* $CaCl_2$ competent *E. coli* DH5α cells (40 μL) in 10% glycerol were removed from –80°C storage and thawed on ice. Once *E. coli* cell

samples thawed, a mixture of target ORF DNA and linearized vector was added, mixed by pipette and briefly centrifuged before being incubated on ice for 30 minutes. Following incubation, samples were heat shocked in a 42°C water-filled heating block for 35 seconds and then held on ice for 2 minutes. RT SOC medium (250 μL) was added to each cell sample followed by incubation at 37°C and 250 rpm for 1 hour. Transformed cells were plated on LB agar plates containing 100 μg/mL carbenicillin and incubated upside down overnight at 37°C. Following incubation, plates were sealed with parafilm and stored at 4°C. Colonies found to have carbenicillin resistance conferred by recombined plasmids were selected for further analysis.

v) PCR Detection of Recombinant Plasmids

Preparation of Template DNA. Following transformation of *E. coli* DH5α with a mixture of the target ORF DNA and the linearized vector, 3 – 4 single colonies from plated putative recombinant *E. coli* cultures were selected and each was inoculated into 10 mL LB broth containing 100 μg/mL carbenicillin. Culture volumes of 10 mL allowed for ample availability for propagation of transformed cultures, short and long term back up storage, as well as DNA extraction. Inoculated broth cultures were incubated over night at 37°C and 250 rpm. Broth cultures (100 μL) were centrifuged at 13,000 ^{x} g for 2 minutes. Pellets were resuspended in 100 μL of 50 mM NaOH and cell suspensions were mixed by vortex for 1 minute and heated at 100°C for 10 minutes. Ten μL of 1 M Tris-HCl (pH 8.0) was added to the samples before additional mixing and centrifugation at 13,000 ^{x} g for 1 minute. Supernatants were used immediately in colony PCR with Taq polymerase.

PCR Reactions. PCR reactions were prepared as follows: 5 μL of $MgCl_2$ free PCR buffer (Invitrogen, USA); 3 μL of 50 mM $MgCl_2$ (Invitrogen, USA); 1 μL of 10 mM dNTPs; 1

μL of 25 μM forward primer (T7 promoter (Sigma, USA)); 1 μL of 25 μM reverse primer (T7 Terminator (Sigma, USA)); 5 μL of Template DNA; 1 μL of Taq DNA polymerase (Invitrogen, USA); 33 μL of sMQH_2O.

Thermocycling Conditions. The following PCR parameters were used: initial denaturation at 94°C for 4 minutes; 35 cycles of denaturation, annealing, and extension at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 3 minutes; final extension at 72°C for 3 minutes; hold at 4°C.

Electrophoresis. Agarose gel (1% w/v) solutions (recipe A2.11) were prepared. Gel solutions were heated by microwave for ~1 minute until the agarose gel powder dissolved completely. Three μL SYBR Safe (10 mg/mL) was added to the solution before being poured into casting frames with up to two rows of 4 – 15 wells and were allowed to solidify. DNA samples were treated with 6X Loading Buffer (recipe A2.4) and loaded into the wells. DNA standards were loaded into separate wells and electrophoresis was run at 120 V for 20 minutes. PCR gel images were taken on the BioRad Gel Documentation System with UV light. *E. coli* colonies containing recombinant plasmids with expected amplicon sizes were selected for downstream analysis.

vi) Recombinant Plasmid Isolation and Sequencing

Plasmid Isolation. Recombinant plasmids containing an insert of expected size were isolated from broth cultures of corresponding colonies using a Minipreparation kit (Qiagen, Germany) according to the manufacturer's instructions. Five mL of each colony broth culture was centrifuged at 16,000 ^{x} g for 3 minutes and the supernatants were discarded. Cells were resuspended in 500 μL of Buffer P1. Buffer P2 (500 μL) was added and the tubes were gently inverted 4 – 6 times without vortex mixing. Buffer N3 (700 μL) was added and the tubes were

inverted gently and immediately 4 – 6 times without vortex mixing. Samples were centrifuged at 16,000 ^{x} g for 10 minutes at 4°C and the supernatants were added to spin columns by pipetting in 800 µL increments. The columns were centrifuged at 16,000 ^{x} g for 1 minute and the flow through was discarded. Wash Buffer PB (500 µL) was added to each column and centrifuged at 16,000 ^{x} g for 1 minute and the flow through was discarded. Wash Buffer PE (750 µL) was added to the columns before centrifugation at 16,000 ^{x} g for 1 minute and the flow through was discarded. The columns were centrifuged at 16,000 ^{x} g for an additional minute and the flow through was discarded. The spin columns were placed in clean 1.5 mL tubes and 50 µL of Elution Buffer EB was added to the columns. Columns were left for a minimum of 1 minute before being spun at 16,000 ^{x} g for 1 minute. Flow throughs were then saved. Concentrations of purified DNA samples were measured on Implen Nanophotometer P330 and purity was determined by A260/A280 nm wavelength ratios. Purified PCR products with a concentration of 50 ng/µL or greater were stored at –20°C until use.

DNA Sequencing and Alignments. Recombinant plasmids that were confirmed to contain an insert of correct size were sent to Genome Quebec (McGill University) for Sanger sequencing using both T7 Promoter (T7P) and T7 Terminator (T7T) primers. A minimum concentration of 50 ng/µL was required for each plasmid sample to be sequenced. Sequencing results were aligned to genomic reference sequences in both directions in .FASTA format using the Multalin open access alignment tool (multalin.toulouse.inra.fr/multalin). Default DNA alignment settings were used for all alignments. Recombinant plasmids containing candidate ORF sequences that aligned with reference sequences were used for downstream *in vitro* protein expression.

III. Protein Expression

i) *In Vitro* Transcription and Translation

Expression of recombinant proteins from candidate ORFs cloned into pIVEX2.3d was performed using a biotechrabbit *In Vitro* Transcription and Translation kit according to the manufacturer's instructions (biotechrabbit, Germany). Protein expression experiments were carried out in an Eppendorf Mastercycler (Eppendorf, Germany) at 30°C for 6 hours and held at 4°C overnight. Expression reaction mixtures were composed of 12 μL reconstituted *E. coli* Lysate, 10 μL reconstituted Reaction Mix, 12 μL reconstituted Amino Acids, 1 μL reconstituted Methionine, 5 μL Reconstitution Buffer and 10 μL of recombinant plasmid DNA. GFP plasmid DNA (10 μL) was included as a positive control. DNA concentrations were required to be a minimum concentration of 50 ng/μL. *In vitro* expression of candidate proteins was analyzed and confirmed by SDS-PAGE and western blotting.

ii) SDS-PAGE

Protein Sample Preparation. Water was brought to a boil in a beaker. Aliquots (15 μL) of recombinant protein samples were mixed by vortex with an equal volume of 2x SDS-PAGE sample buffer #2 (recipe A2.13). Mixed samples were incubated in the boiling water for 10 minutes and mixed by vortex every 2 minutes. Samples were allowed to cool to RT before immediate usage or storage at –20°C long-term.

Preparation of Polyacrylamide Gels. Separating gel solutions (recipe A2.14) were poured into the casting frames and allowed to set for 1 hour at RT beneath an aqueous upper phase. Following polymerization, the upper phases were poured off and solidified gels were rinsed thrice. Excess water was removed by capillary action using a Kimwipe (Kimberly-Clark, USA). Stacking gel solutions (recipe A2.15) were poured on top of solidified separating gels in the casting frames and well combs inserted into the solutions immediately. Stacking gel solutions

were allowed to solidify for 45 minutes. Gels were used immediately or wrapped in damp paper towel and stored at 4°C in bags to maintain humidity until use.

Electrophoresis. Casting frames containing separating and stacking gels were placed on electrode stands and submersed in 1x SDS-PAGE Running Buffer (recipe A2.16) in the electrophoresis Mini Tank (BioRad, USA). Each prepared protein sample (15 μL) was added to the wells along with prestained low-range protein standards (BioRad, USA) in the first well. Electrophoresis was run at 200 V for 45 minutes. SDS-PAGE gels were removed for downstream transfer to nitrocellulose membranes and western blot analysis.

iii) Immunoblotting

Protein Transfer. Protein transfer buffer (recipe A2.17) was warmed to RT from 4°C. Polyacrylamide gels were removed from their SDS-PAGE casting frames following electrophoresis and stacking gels were cut off from the separating gels. Two blotting papers (BioRad, USA) and one nitrocellulose membrane (BioRad, USA), per separating gel, were soaked in protein transfer buffer (recipe A2.17) and assembled in a Trans-Blot Turbo Transfer System (BioRad, USA) with air bubbles removed using a roller. The Trans-Blot Turbo Transfer System (BioRad, USA) was run at 15 V for 30 minutes.

Probing Western Blots. Western blotting assays were carried out using the iBind Automated Western System (Life Technologies, USA). Nitrocellulose membranes (BioRad, USA) containing transferred expressed proteins were immersed in 5 mL of 1x iBind Solution (recipe A2.18). iBind Cards (Life Technologies, USA) were placed on the iBind stage (Life Technologies, USA) and 5 mL of 1x iBind Solution were spread evenly across each. 1x iBind Solution (1 mL) was pooled in the middle of each iBind Card and the nitrocellulose membranes containing transferred proteins were placed directly on top of the pooled solution, protein-side

down, with air bubbles removed using a roller. The iBind Western System apparatus was sealed and wells 1 – 4 were filled sequentially with 2 mL of diluted 1° antibody (recipe A2.19), 2 mL of iBind Solution, 2 mL of diluted 2° antibody (recipe A2.20) and 6 mL iBind solution, respectively. The wells were closed and iBind western blot assays were run for 2.5 hours to overnight according to specifications. Membranes were then rinsed before proceeding to immunodetection.

Immunodetection. Colour development substrate solutions were prepared by combining 9 mL MQH_2O, 1 mL of 10x HRP Color Development Buffer (BioRad, USA), 2 mL Color Reagent A (BioRad, USA), and 60 μL of Color Reagent B (BioRad, USA) for each nitrocellulose membrane following western blotting. For colour development, membranes were incubated in substrate solutions for 10 minutes at RT on a Belly Dancer Shaker (IBI Scientific, USA). Membranes were rinsed with MQH_2O and images taken on the BioRad Gel Documentation System. Protein expression samples confirmed to contain recombinant proteins by immunoblotting with anti-His antibody were saved at 4°C until use in downstream purification within a week.

2.1.5 Protein Purification

Recombinant proteins demonstrated to have been expressed successfully through western blotting were purified by affinity chromatography with Ni-NTA agarose (Qiagen, Germany). Ten mL of Ni-NTA agarose slurry (Qiagen, Germany) was poured into 15 mL tubes and beads allowed to settle for 10 minutes. Slurries were adjusted to 50% (v/v) with Wash/Equilibrium Buffer (WEB) (pH 8.0) (recipe A2.21) centrifuged for 5 minutes at 200 ^{x} g, and the liquid portions discarded. The slurry was then adjusted to 50% (v/v) with WEB, centrifuged at 200 ^{x} g, and the liquid portion was discarded. This process was repeated a total of three times. The washed slurry was then adjusted to 50% (v/v) with WEB and vortexed before 100 μL volumes were aliquoted into filter wells (AcroPrep Advanced 96-Well Filter Plates for Aqueous Filtration (Pall, USA)) in Non-sterile, 96-Well Non-binding Microplates, (Greiner Bio-One, Austria). Recombinant protein samples were diluted in a 1:1 ratio with WEB. Filter plates were stacked on top of collection plates and 100 μL of each diluted protein sample was added to separate wells. Filter plates were incubated at RT for 15 minutes at 200 rpm on a Plate Shaker (Thermo Scientific, USA). Plate assemblies were centrifuged for 1 minute at 1,000 ^{x} g, to allow other *E. coli* proteins to pass into collection plate wells, and flow throughs were discarded from the collection plate wells. WEB (250 μL) was added to filter plate wells containing Ni-NTA agarose bound recombinant proteins and centrifuged at 1,000 ^{x} g for 1 minute, and flow throughs were discarded from the collection plate wells. The process was repeated as above, three times total. Filter plates were placed on new collection plates and 35 μL of Elution Buffer B (pH 8.0) (recipe A2.22) was added to each of the protein sample wells and plate assemblies were incubated at RT for 5 minutes at 300 rpm on the Plate Shaker. The plate assembly was centrifuged at 1,000 ^{x} g for 1 minute to collect the eluted protein samples in collection plate wells. Elution was repeated a

total of two times. Collection plates were sealed and stored at 4°C until use. Purified proteins were quantified on Qubit (Invitrogen, USA) according to the Manufacturer's Qubit 3.0 Fluorometer Protein Assay protocol. Qubit Working Solutions (WS) were prepared with 199 μL Qubit Protein Buffer (Invitrogen, USA) and 1 μL Qubit Protein Reagent (Invitrogen, USA). Five μL of each protein sample suspended in purification Elution Buffer B and a blank control of 5 μL Elution Buffer B were diluted in 195 μL of WS. Ten μL each of 3 Protein Standards (Invitrogen, USA) of 0 ng/μL, 200 ng/μL, and 400 ng/μL were diluted in 190 μL of WS separately. Candidate protein, control, and protein standard samples were mixed by vortex for 3 seconds, incubated in the dark at RT for 15 minutes, and quantification measurements of the samples were taken against the standards within 10 minutes of incubation.

2.1.6 Immunoreactivity Analysis

Protein Arrays. Nitrocellulose membranes (BioRad, USA) were cut into 6x9 cm sheets and 14 square grids were added with pencil. Three μL of each Ni-NTA agarose purified recombinant proteins, of at least 30 ng/μL, were spotted onto nitrocellulose membrane grid spots by pipette. Onto each protein array, 3 μL of 20 mg/mL *Brucella* crude LPS (112) were spotted as positive control and 3 μL of Classical Swine Fever Virus (CSFV) E2 protein (113) as negative control. All spots were allowed to dry completely for 10 – 15 minutes. CSFV E2 protein and *Brucella* LPS samples were sourced from archived, in-house isolations suspended in phosphate buffered saline (PBS) and 50% (v/v) glycerol.

Western Blotting. Western blotting assays were carried out using the iBind Automated Western System (Life Technologies, USA) as instructed by the manufacturer. Nitrocellulose membranes (BioRad, USA) containing spotted protein candidates and controls were immersed in 5 mL of 1x iBind Solution (recipe A2.18). iBind Cards (Life Technologies, USA) were placed on the iBind stage (Life Technologies, USA) and 5 mL of 1x iBind Solution (recipe A2.18) was spread evenly across each. One mL of 1x iBind Solution was pooled in the middle of each iBind Card and the nitrocellulose membranes containing transferred proteins were placed directly on top of the pooled solution protein-side down with air bubbles removed using a roller. The iBind western system apparatus was sealed and wells 1 – 4 were filled sequentially with 2 mL of diluted bovine serum as the 1° antibody (recipe A2.23), 2 mL of 1x iBind Solution, 2 mL of diluted 2° antibody (recipe A2.24) and 6 mL 1x iBind solution, respectively. The wells were closed and the iBind western blot system assays were allowed to run for 2.5 hours to overnight, according to specifications. Membranes were then rinsed before proceeding to immunodetection.

Chemiluminescence. BioRad's Clarity Enhanced Chemiluminescence (ECL) reagents were used according to the manufacturer's instructions. Following antibody probing, nitrocellulose membranes (BioRad, USA) were rinsed and kept moist in MQH_2O. Clarity Western Peroxide Reagent (BioRad, USA) and Clarity Western Luminol/Enhancer Reagent (BioRad, USA) were combined in a 1:1 (v/v) ratio solution and each membrane was immersed in 7 mL of the 1:1 solution and incubated at RT for 5 minutes with occasional agitation. ECL results were imaged on a BioRad Gel Documentation System using the chemiluminescence filter. Image capture was programmed to occur every 30 seconds for 3 minutes to track the intensity of all spots during the HRP reaction.

3. Results

3.1 Candidate Selection, Production, and Evaluation

3.1.1 Protein Candidate Selection

Pools of protein candidates and corresponding genomic information encoded by each chromosome of *B. abortus* bv. 2 str. 86/8/59 were generated through the application of bioinformatics to its whole genome reference sequence. Proteins coded by the ORFs from chromosomes 1 and 2 of the *B. abortus* bv. 2 str. 86/8/59 genome were identified according to the selection criteria and are shown in Table 1 and Table 2, respectively. As seen in the results, all protein candidates selected were predicted to have localization extracellularly or in the CE. Many of the proteins selected possessed a positive signal peptide in their amino acid sequences deduced from the ORFs and all of the Protein IDs were determined to be unique to the *Brucella* genus. Results shown in Table 1 and Table 2 were obtained through PSORTb module outputs from the genomic analysis as well as from cross-referencing protein candidate information with the NCBI GenBank database. The 101 selected candidates presented in Table 1 and Table 2 were anticipated to have the potential for immunoreactivity against *B. abortus*-infected bovine sera. This hypothesis was based on bioinformatic data predicting that these proteins possess characteristics conducive to immunogenicity. There are 50 candidates presented in Table 1 and 51 candidates presented in Table 2.

Table 1: Selected Chromosome 1 Protein Candidates. Candidates identified in PSORTb from *B. abortus* bv. 2 str. 86/8/59 genome (Accession number: NZ_CP007765.1). Candidate discovery parameters focused on extracellular or CE localization and presence of a signal peptide. Signal Peptide Detected: "+" and "–" indicate detection or not, respectively. Gene size displayed in base pairs (bp). Protein size displayed in amino acids (aa). Predicted Cellular Localization: "C" indicates cytoplasmic membrane; "E" indicates extracellular; "P" indicates periplasmic. Identical Protein Search: "/" indicates the protein is specific to the *Brucella* genus.

Protein Accession Number (NCBI)	Protein ID (NCBI)	Genomic Locus Tag (NCBI)	Signal Peptide Detected (SignalP)	Gene Size (bp)	Protein Size (aa)	Predicted Cellular Localization (PSORTb)	Identical Protein Search (NCBI)
AIJ93673	WP_002964760	DK55_1636	+	960	319	C	/
AIJ93099	WP_002969561	DK55_1316	+	2577	858	C	/
AIJ92748	WP_002963639	DK55_513	+	2895	964	C	/
AIJ91845	WP_002964966	DK55_1840	+	1266	421	C	/
AIJ92038	WP_002975817	DK55_1488	+	756	251	C	/
AIJ92635	WP_002964334	DK55_1204	+	585	194	C	/
AIJ93603	WP_002965376	DK55_143	–	837	278	E	/
AIJ91927	WP_002971034	DK55_1082	–	945	314	E	/
AIJ93481	WP_002965213	DK55_2081	–	690	229	E	/
AIJ93502	WP_002969455	DK55_571	+	963	320	P	/
AIJ92906	WP_002966934	DK55_1599	+	990	329	P	/
AIJ93147	WP_002965356	DK55_125	+	1029	342	P	/
AIJ91981	WP_002966964	DK55_1735	–	1116	371	P	/
AIJ92065	WP_002966965	DK55_1737	+	1116	371	P	/
AIJ92184	WP_002964329	DK55_1200	+	1446	481	P	/
AIJ92709	WP_002971565	DK55_453	–	612	203	P	/
AIJ92265	WP_002965189	DK55_2058	–	987	328	P	/

AIJ93597	WP_002965136	DK55_2006	–	492	163	P	/
AIJ92545	WP_002964757	DK55_1634	+	843	280	P	/
AIJ92372	WP_002965328	DK55_96	+	504	167	P	/
AIJ93692	WP_002966801	DK55_958	+	534	177	P	/
AIJ92714	WP_002964222	DK55_1103	+	591	196	P	/
AIJ92550	WP_002967079	DK55_116	+	612	203	P	/
AIJ92062	WP_002964734	DK55_1611	–	822	273	P	/
AIJ92115	WP_002966680	DK55_345	+	843	280	P	/
AIJ93181	WP_002965019	DK55_1890	+	846	281	P	/
AIJ93242	WP_011265364	DK55_1514	+	873	290	P	/
AIJ92270	WP_002965502	DK55_267	–	882	293	P	/
AIJ92208	WP_002964447	DK55_1324	+	1005	334	P	/
AIJ92703	WP_002964786	DK55_1663	–	1071	356	P	/
AIJ92314	WP_002969645	DK55_35	–	1083	360	P	/
AIJ93060	WP_002969454	DK55_573	+	1098	365	P	/
AIJ92581	WP_016769635	DK55_1578	+	1104	367	P	/
AIJ92371	WP_002971406	DK55_1337	+	1116	371	P	/
AIJ93725	WP_002966791	DK55_922	+	1215	404	P	/
AIJ93499	WP_002964785	DK55_1662	+	1332	443	P	/
AIJ92890	WP_002964336	DK55_1206	+	1425	474	P	/
AIJ93662	WP_002964503	DK55_1380	+	1575	524	P	/
AIJ92858	WP_002965257	DK55_33	+	1869	622	P	/
AIJ93619	WP_002969536	DK55_275	–	2121	706	P	/
AIJ92061	WP_002964144	DK55_103	–	573	190	P	/
AIJ93027	WP_002963716	DK55_594	–	600	199	P	/

AIJ93253	WP_002969429	DK55_723	+	690	229	P	/
AIJ93047	WP_002964581	DK55_1457	+	753	250	P	/
AIJ93661	WP_002964104	DK55_994	–	759	252	P	/
AIJ92992	WP_002971474	DK55_874	+	789	262	P	/
AIJ92394	WP_002969426	DK55_762	–	801	266	P	/
AIJ93639	WP_002964174	DK55_1059	–	855	284	P	/
AIJ91931	WP_002969473	DK55_422	–	1290	429	P	/
AIJ92261	WP_002965256	DK55_32	+	1848	615	P	/

Table 2: Selected Chromosome 2 Protein Candidates. Candidates identified in PSORTb from *B. abortus* bv. 2 str. 86/8/59 genome (Accession number: NZ_CP007764.1). Candidate discovery parameters focused on extracellular or CE localization and presence of a signal peptide. Signal Peptide Detected: "+" and "–" indicate detection or not, respectively. Gene size displayed in base pairs (bp). Protein size displayed in amino acids (aa). Predicted Cellular Localization: "E" indicates extracellular; "O" indicates outer membrane; "P" indicates periplasmic. Identical Protein Search: "/" indicates the protein is specific to the *Brucella* genus.

Protein Accession Number (NCBI)	Protein ID (NCBI)	Genomic Locus Tag (NCBI)	Predicted Signal Peptide (SignalP)	Gene Size (bp)	Protein Size (aa)	Predicted Cellular Localization (PSORTb)	Identical Protein Search (NCBI)
AIJ91379	WP_002966373	DK55_2199	–	882	293	E	/
AIJ91259	WP_002965683	DK55_2264	–	885	295	E	/
AIJ90845	WP_002967184	DK55_2154	–	729	242	O	/
AIJ91125	WP_002965726	DK55_2306	+	852	284	O	/
AIJ91039	WP_002972093	DK55_2515	+	522	173	P	/
AIJ91095	WP_002966661	DK55_3027	+	1005	334	P	/
AIJ90972	WP_002965896	DK55_2475	+	1236	411	P	/
AIJ90995	WP_002967274	DK55_2525	+	1266	421	P	/
AIJ91341	WP_002966282	DK55_2847	+	1266	421	P	/
AIJ91201	WP_002965982	DK55_2557	+	1302	433	P	/
AIJ90882	WP_002967374	DK55_2918	+	1479	492	P	/
AIJ90867	WP_002966234	DK55_2804	–	1500	499	P	/
AIJ91442	WP_002965856	DK55_2438	+	1614	537	P	/
AIJ91734	WP_002967358	DK55_2874	+	1920	639	P	/
AIJ91528	WP_002967354	DK55_2867	+	441	146	P	/
AIJ91673	WP_002966084	DK55_2659	+	546	181	P	/
AIJ91269	WP_002966451	DK55_2127	–	732	243	P	/

AIJ90810	WP_002966490	DK55_3191	+	756	251	P	/
AIJ90813	WP_002966007	DK55_2581	+	762	253	P	/
AIJ90800	WP_002965959	DK55_2536	–	774	257	P	/
AIJ91347	WP_002971234	DK55_2580	+	774	257	P	/
AIJ90704	WP_002966428	DK55_2149	–	789	262	P	/
AIJ91026	WP_002965837	DK55_2421	+	789	262	P	/
AIJ91445	WP_006091569	DK55_2422	–	807	268	P	/
AIJ91223	WP_002967364	DK55_2886	–	852	283	P	/
AIJ91474	WP_002965965	DK55_2542	+	930	309	P	/
AIJ91202	WP_002965789	DK55_2366	+	948	315	P	/
AIJ91116	WP_002967145	DK55_3087	+	981	326	P	/
AIJ90955	WP_002971263	DK55_2775	+	990	329	P	/
AIJ90792	WP_002965941	DK55_2519	+	1020	339	P	/
AIJ91123	WP_002967211	DK55_2279	+	1020	339	P	/
AIJ91288	WP_002966632	DK55_3057	+	1020	339	P	/
AIJ91656	WP_002971932	DK55_3004	+	1029	342	P	/
AIJ91360	WP_002967344	DK55_2829	+	1047	348	P	/
AIJ91621	WP_002966326	DK55_2887	+	1131	376	P	/
AIJ90806	WP_002966472	DK55_2105	–	1137	378	P	/
AIJ91520	WP_002966193	DK55_2765	+	1167	388	P	/
AIJ91663	WP_002965989	DK55_2564	+	1173	390	P	/
AIJ91666	WP_002967329	DK55_2778	+	1200	399	P	/
AIJ90905	WP_002966215	DK55_2786	+	1212	403	P	/
AIJ91068	WP_002966640	DK55_3050	+	1293	430	P	/
AIJ90888	WP_002966309	DK55_2873	+	1395	464	P	/

AIJ91738	WP_002967388	DK55_2991	+	1545	514	P	/
AIJ91135	WP_002967389	DK55_2997	+	1557	518	P	/
AIJ91664	WP_002966094	DK55_2667	+	1578	525	P	/
AIJ91247	WP_002966198	DK55_2768	+	1584	527	P	/
AIJ91268	WP_002965937	DK55_2514	+	1605	534	P	/
AIJ91153	WP_002968915	DK55_2984	+	1620	539	P	/
AIJ91182	WP_002969381	DK55_2949	+	945	314	P	/
AIJ91222	WP_002967300	DK55_2632	+	1848	615	P	/
AIJ90956	WP_002971934	DK55_2990	–	2286	761	P	/

3.1.2 Design of Oligonucleotide Primers for ORF Cloning

To produce candidate ORFs by PCR, custom oligonucleotide primers were designed using the Primer Design function on SeqBuilder. Designed primer sets are shown in Table 3 and Table 4. Nucleotide sequences added to the 5' ends of designed primers, that include a 6x His-tag coding sequence and have homology with the pIVEX2.3d cloning vector, are displayed on each of the two primers for every ORF candidate. The sequences added to the primers were 26 nucleotides long on the 5' end of the forward primers and 25 nucleotides long on the 5' end of the reverse primers. Primers ranged from 15 – 30 bp in length prior to inclusion of the added terminal sequences that are homologous to the MCS on the pIVEX2.3d cloning vector. Forward primers presented in Table 3 and Table 4 underwent further modifications including adjusting start codons to a uniform ATG sequence. Additionally, stop codons were removed from all reverse primers.

Table 3: Oligonucleotides Designed for Selected Chromosome 1 Protein Candidate Genes. Oligonucleotides for use as primers in downstream PCR amplification displayed. Red nucleotides are reverse complement to the MCS in NdeI linearized pIVEX2.3d cloning vectors. Black nucleotides are reverse complement to the terminal regions of candidate genes. Forward and reverse primer sequences are shown for each candidate.

Candidate Accession Number (NCBI)	Primer Direction	Oligonucleotide Sequences for Candidate Genes
AIJ92906	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAAAACTATTGATCGC
	Reverse	GATGATGATGATGATGAGAACCCCCCTTGCGGCTTTCCAGAAG
AIJ93147	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAGGTTATCGAAGTTTTCTTT
	Reverse	GATGATGATGATGATGAGAACCCCCTTTGCCGGGTTTATAT
AIJ91981	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGAAATCCTTATTTTGCGG
	Reverse	GATGATGATGATGATGAGAACCCCCCTTCTGGAAGTAATTATACT
AIJ92065	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAGAAAAACACTTTTTTCGG
	Reverse	GATGATGATGATGATGAGAACCCCCCTGCTGAATGTAATTATACT
AIJ92184	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCCCAGGAAATTCACCCG
	Reverse	GATGATGATGATGATGAGAACCCCCCTTCCGCCTGCAACTGCAAGG
AIJ93502	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCGAAGGAATTTGATGAAG
	Reverse	GATGATGATGATGATGAGAACCCCCCGAATGGAGAATCTGGGA
AIJ93099	Forward	GTTTAACTTTAAGAAGGAGATATACCTTGATCCCTTTATCGACATTCCG
	Reverse	GATGATGATGATGATGAGAACCCCCCTTACGCACGATCGTCCTTGTCA
AIJ92748	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAGAGGTTTTTCAAAAAACTG
	Reverse	GATGATGATGATGATGAGAACCCCCCTCACGCCGCCGCATCCA
AIJ93673	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCGCCCTGTCCTGAAC
	Reverse	GATGATGATGATGATGAGAACCCCCCTTACTCCGCAGCGTGCAGC
AIJ92038	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAACTGTCGGCAAGA
	Reverse	GATGATGATGATGATGAGAACCCCCCTTACCGCCTGCCGCCGAGACGCA
AIJ91845	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCTCATAACAGGAAGAAACAA

	Reverse	GATGATGATGATGATGAGAACCCCCTCACTGCACCGCCTCCCCATG
AIJ92709	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACAAATTCCAGCGACGATTTC
	Reverse	GATGATGATGATGATGAGAACCCCCTCAGGGATAAAGCCTGTCCT
AIJ92265	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTTCGATGGGGTGTA
	Reverse	GATGATGATGATGATGAGAACCCCCTCACACAGTCTCGCTCACCCC
AIJ93597	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAGTGATAAGGCCGCGGGCGAA
	Reverse	GATGATGATGATGATGAGAACCCCCTCAGCTCTTCACAGCGGAC
AIJ92635	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGCGCGTTTTGTTT
	Reverse	GATGATGATGATGATGAGAACCCCCACTGAAGCCTCCGCATGAGC
AIJ92714	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTCTTTCATTCGCTCGGCG
	Reverse	GATGATGATGATGATGAGAACCCCCCTTCGTGTCAGCTTCG
AIJ92545	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAAGACTTCTCGCCACCAC
	Reverse	GATGATGATGATGATGAGAACCCCCTTCAGCGGCGCCGTG
AIJ93692	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCGCGTATTGAAGCGTATC
	Reverse	GATGATGATGATGATGAGAACCCCCAGCCCCTCGATCCCATGC
AIJ92372	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCTTGGAAAATCGATCGTCGCAGC
	Reverse	GATGATGATGATGATGAGAACCCCCTTTGCACTTCTGGATC
AIJ92062	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGATGGTGATAAAATGATGA
	Reverse	GATGATGATGATGATGAGAACCCCCCGCTGCGGCCCATCCCAT
AIJ93603	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGAAACGATCTCAACCGC
	Reverse	GATGATGATGATGATGAGAACCCCCGCTGCGCAGCCGTTCCGA
AIJ92115	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAATCCTGTCTGTTGAGCCTGACGA
	Reverse	GATGATGATGATGATGAGAACCCCCATAAAAGCTTGCCGGG
AIJ92550	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACCGAAACAACCGAAAAACC
	Reverse	GATGATGATGATGATGAGAACCCCCTCGTTGTTGAAGAGCCT
AIJ93181	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCATAAGAAGAAAATTCTTG
	Reverse	GATGATGATGATGATGAGAACCCCCGCGCGCACCGTCCGGCA
AIJ93242	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGGACGTAACGATGAAAA

	Reverse	GATGATGATGATGATGAGAACCCCCGTGCTTCACATCCGACCA
AIJ92270	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACAGCGGCCAGAGAGATTAC
	Reverse	GATGATGATGATGATGAGAACCCCCTGCTGCCGCACCTTTCA
AIJ91927	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGTGCTTGCATGGAGCCAG
	Reverse	GATGATGATGATGATGAGAACCCCCCCCCATCACGCCTTG
AIJ92208	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAGAAAAATTATTCTATACG
	Reverse	GATGATGATGATGATGAGAACCCCCCTTGCCGGTAAAGAGTT
AIJ92703	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGGCTGGCCTGACATCTT
	Reverse	GATGATGATGATGATGAGAACCCCCGAACATTTCGCTTGGGTCG
AIJ92314	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTTCGGGGCTGTGCG
	Reverse	GATGATGATGATGATGAGAACCCCCTTCAAGACCGACGATCT
AIJ93060	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACGATTGAAGCCAAAATCAA
	Reverse	GATGATGATGATGATGAGAACCCCCAAGCTTAACCCAACCAC
AIJ92581	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGGGATCAAATCCTTCCTTC
	Reverse	GATGATGATGATGATGAGAACCCCCCTGGCCGGTGACGATCTTGG
AIJ92371	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGCTGTTATAGCAGGCTTGAT
	Reverse	GATGATGATGATGATGAGAACCCCCGATTTTCTTCCACTGGC
AIJ93725	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGATTTACAAAGCCATTATA
	Reverse	GATGATGATGATGATGAGAACCCCCTCCGCCGCTGAGATTTGA
AIJ93499	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAATCGGCATCATCAATAC
	Reverse	GATGATGATGATGATGAGAACCCCCTTCGAGCAGCGGCGACCAG
AIJ92890	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAATTGGCGGTGTTTGGG
	Reverse	GATGATGATGATGATGAGAACCCCCGCGCACGAACTGGC
AIJ93662	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGCAATTGCACCAAAGGC
	Reverse	GATGATGATGATGATGAGAACCCCCGTCGATACGGATCGTCACGA
AIJ92858	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAGCTTTTTGGGTGAGT
	Reverse	GATGATGATGATGATGAGAACCCCCTGCACCGCCGGTCTTGAGCT
AIJ93619	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACACCTATCGGCAACAGAA

	Reverse	GATGATGATGATGATGAGAACCCCCTTCGAAATCACCAGTCTGCG
AIJ92061	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACGAAGATTGTATTCGTATCC
	Reverse	GATGATGATGATGATGAGAACCCCCTCAGTTCTGGCGTTCGGGCACC
AIJ93027	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGCTTTCGAACTGCCC
	Reverse	GATGATGATGATGATGAGAACCCCCTTATGCCGCCTTTTCGTACA
AIJ93481	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAATACTTGCCCTCG
	Reverse	GATGATGATGATGATGAGAACCCCCTTATTCTCCGCCCTGCCG
AIJ93253	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCTGCGCACCTTGGCAGC
	Reverse	GATGATGATGATGATGAGAACCCCCCTATTGCATTGCCGGCATGATGA
AIJ93047	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAACACTCGTGCTAGCAATT
	Reverse	GATGATGATGATGATGAGAACCCCCTTACTTGATTTCAAAAACGACAT
AIJ93661	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGCTAAGGGAACCTTTGCC
	Reverse	GATGATGATGATGATGAGAACCCCCTCATGCCTCTTTCCTCACCC
AIJ92992	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCGCGGTTTTAAGCGTATTGC
	Reverse	GATGATGATGATGATGAGAACCCCCTCAGTTTTCAGCGCCGTAAGC
AIJ92394	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGTTCACTCCTCTCAATGC
	Reverse	GATGATGATGATGATGAGAACCCCCTTAGCGCACGGGGATGCC
AIJ93639	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACCGAGAAAAGCGCATTCGTC
	Reverse	GATGATGATGATGATGAGAACCCCCTCACTCGGCTTCGCCAGTCA
AIJ91931	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCGTCTTGTATCCTTACGAGC
	Reverse	GATGATGATGATGATGAGAACCCCCGCTCCAGCCGCCGCCATA
AIJ92261	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACATTCCTTTGCATGAA
	Reverse	GATGATGATGATGATGAGAACCCCCTGCCTTAATCCACCACAGCG

Table 4: Oligonucleotides Designed for Selected Chromosome 2 Protein Candidate Genes. Oligonucleotides for use as primers in downstream PCR amplification displayed. Red nucleotides are reverse complement to the MCS in NdeI linearized pIVEX2.3d cloning vectors. Black nucleotides are reverse complement to the terminal regions of candidate genes. Forward and reverse primer sequences are shown for each candidate.

Candidate Accession Number (NCBI)	Primer Direction	Oligonucleotide Sequences for Candidate Genes
AIJ91528	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCGTAATACCGCAATCAAGTT
	Reverse	GATGATGATGATGATGAGAACCCCCCTTCACTTCTGCAAGGATTTT
AIJ91039	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGTCCTTATTTATT
	Reverse	GATGATGATGATGATGAGAACCCCCTTCGATCACGCCGCAGG
AIJ91673	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACCTTTAAACCGGAAAA
	Reverse	GATGATGATGATGATGAGAACCCCCCTTAACGGGGATCGC
AIJ90845	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAAACAGCATGAACAAAG
	Reverse	GATGATGATGATGATGAGAACCCCCGAAAGGAGAAAACTGGTCG
AIJ91269	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCAGAACAATTCGATC
	Reverse	GATGATGATGATGATGAGAACCCCCTCCGTTTTTGGAACCCAG
AIJ90810	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGAAGCTGATCGCCACA
	Reverse	GATGATGATGATGATGAGAACCCCCCTCGGCAGCCTCCGCAG
AIJ90813	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCTAAAGGCCATAGTATTCGC
	Reverse	GATGATGATGATGATGAGAACCCCCATAGATATCGAAATCGAAAT
AIJ91347	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTGAAATCACTCGTATTGG
	Reverse	GATGATGATGATGATGAGAACCCCCCATAGATATCGAAATCGAAAT
AIJ90800	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAATTCGCTCAGATTCT
	Reverse	GATGATGATGATGATGAGAACCCCCCTTGGAAACGTCCTGGC
AIJ91026	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTGACGGATAGGTTGCGC
	Reverse	GATGATGATGATGATGAGAACCCCCCTAGATGCCCATTTTCTGGA
AIJ90704	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAGCCCTGACGATTG

	Reverse	GATGATGATGATGATGAGAACCCCCACGCAAGTTCTTCGAT
AIJ91445	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGGCATCTATAATACCGAG
	Reverse	GATGATGATGATGATGAGAACCCCCGTTCGCCTTCGGGGTCA
AIJ91223	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGCAAAGGTCGCAGGAAAAC
	Reverse	GATGATGATGATGATGAGAACCCCCAGACGTAAAATCCTTCAAC
AIJ91379	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGATAAGGCCCTGTCGTCA
	Reverse	GATGATGATGATGATGAGAACCCCCAAGATCAGGCAATTTTTCCG
AIJ91474	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAGGATCGAATATCATGCTT
	Reverse	GATGATGATGATGATGAGAACCCCCCTTGCTCTTGTCGAAAG
AIJ91182	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGCTCAGAAATCCGACTCC
	Reverse	GATGATGATGATGATGAGAACCCCCTTTTGTGCCGTAGGTGAC
AIJ91202	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTCAAGAAGGGTATGCGCG
	Reverse	GATGATGATGATGATGAGAACCCCCCTGTTCCAGAACGAACGGC
AIJ91116	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAACGTTTTTCCAGTGCACTT
	Reverse	GATGATGATGATGATGAGAACCCCCACGGCGCGGCAGCAATCGT
AIJ90955	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCTTGGCGTAGGTATCA
	Reverse	GATGATGATGATGATGAGAACCCCCTGCCTCGACCGCCACTTCCC
AIJ90792	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTCCATCATTGCGCGTT
	Reverse	GATGATGATGATGATGAGAACCCCCATTGTCGAAGCCGACTTTGT
AIJ90806	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAACATCGCGATTATCGGCAC
	Reverse	GATGATGATGATGATGAGAACCCCCGAAGCTGCTTTTCATCCTGT
AIJ90867	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACAGATCGCCCGATAAT
	Reverse	GATGATGATGATGATGAGAACCCCCTTCCGCTGCAGTAATTTTT
AIJ90882	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGGTAATTTACTGAAAGC
	Reverse	GATGATGATGATGATGAGAACCCCCTTCAGCCCAATAGGCCTGGG
AIJ90888	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGTTCTCCCCCGCCTGTT
	Reverse	GATGATGATGATGATGAGAACCCCCGTCATTGTGCTTGCTTT
AIJ90905	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAGACTGTCCAGGCTCCTTAT

	Reverse	GATGATGATGATGATGAGAACCCCCTTTCTTGAAGCGCATCTTGG
AIJ90956	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAACGTCGCGAGTGC
	Reverse	GATGATGATGATGATGAGAACCCCCAGATGGCTGCATAATCAGGT
AIJ90972	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTGATCAGAAAATGGAAGGC
	Reverse	GATGATGATGATGATGAGAACCCCCTTTCTTCAAGATGGCGTCTG
AIJ90995	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCATAAACTTCTGAAACTTG
	Reverse	GATGATGATGATGATGAGAACCCCCTTTTGCGGCTTCAACCG
AIJ91068	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGCGGCCTGCCTTGC
	Reverse	GATGATGATGATGATGAGAACCCCCTTCAACGGTCTTTCCTAACA
AIJ91095	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAAATCTGCATTCCTTG
	Reverse	GATGATGATGATGATGAGAACCCCCTTTCGGCAGGCAGTCTTTCA
AIJ91123	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAAAAGACAGTTTGGGGTG
	Reverse	GATGATGATGATGATGAGAACCCCCTTTGACCGGCGGGGCGTACA
AIJ91135	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCTACATCTGGGAACATT
	Reverse	GATGATGATGATGATGAGAACCCCCCTTGGCCTGTCCGATTTCCC
AIJ91153	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCTATTCAAGACAACGGG
	Reverse	GATGATGATGATGATGAGAACCCCCTCTGGATACGGAAAGC
AIJ91201	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTCACCCGTCTGATCAC
	Reverse	GATGATGATGATGATGAGAACCCCCTTGAGCTGCGGCGATTG
AIJ91222	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTGAACAGATTTATTGCT
	Reverse	GATGATGATGATGATGAGAACCCCCTTGGGACGTGTGCCAC
AIJ91247	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAGGTTGCGTAATTTTTATTC
	Reverse	GATGATGATGATGATGAGAACCCCCTTCGAGTATCGCCCATTTTT
AIJ91268	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACTGGAATTACCCGC
	Reverse	GATGATGATGATGATGAGAACCCCCAACGGTCACGAATTGCG
AIJ91288	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAACGTCGTAATTTTCTGGC
	Reverse	GATGATGATGATGATGAGAACCCCCTTTCCCTTCAATCTGTTCCT
AIJ91341	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAGGGTTTTGACGGGAGCACT

	Reverse	GATGATGATGATGATGAGAACCCCCCCAGCCCGAGCCTTTAAGC
AIJ91360	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGTTTGCTCGCCTTGCCC
	Reverse	GATGATGATGATGATGAGAACCCCCTTGCGCCAGCCAGG
AIJ91442	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTCTGGGTTTGTTGCGTC
	Reverse	GATGATGATGATGATGAGAACCCCCTGGTTCCAGCCATGTGTC
AIJ91520	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTTCAGTCTTATTCGCGCAAA
	Reverse	GATGATGATGATGATGAGAACCCCCGTTTTCGACCGAGAGGGC
AIJ91621	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGCCGACCAGATACAAGTCA
	Reverse	GATGATGATGATGATGAGAACCCCCCATCCCCGACGGCGCCAGT
AIJ91656	Forward	GTTTAACTTTAAGAAGGAGATATACCATGTCCAAGAAAATAAAAACGAT
	Reverse	GATGATGATGATGATGAGAACCCCCTTCGGCCAGCCAGTTTTG
AIJ91663	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAGAAAATTGCGCTCACCG
	Reverse	GATGATGATGATGATGAGAACCCCCCTGCGCTTTCAGGCCCGGAT
AIJ91664	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGTTCGCGGAATTTTGATG
	Reverse	GATGATGATGATGATGAGAACCCCCCGTTCTTGACGGATAGCCAGC
AIJ91666	Forward	GTTTAACTTTAAGAAGGAGATATACCATGGAAAAACATCTTATCGCAT
	Reverse	GATGATGATGATGATGAGAACCCCCCTGCGGCAACCGGATTTCC
AIJ91734	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACTGAAGAAACCAGGAAA
	Reverse	GATGATGATGATGATGAGAACCCCCGGCCTTTTTCGGTTCG
AIJ91738	Forward	GTTTAACTTTAAGAAGGAGATATACCATGACAATTCGCACCTTGTTCAA
	Reverse	GATGATGATGATGATGAGAACCCCCCTGTTTCTTCAGCCAGACG
AIJ91125	Forward	GTTTAACTTTAAGAAGGAGATATACCATGAAACGCGGTTGCGCCATT
	Reverse	GATGATGATGATGATGAGAACCCCCGCGGAACTCGTAACG
AIJ91259	Forward	GTTTAACTTTAAGAAGGAGATATACCATGCTGCAATTGGCGATG
	Reverse	GATGATGATGATGATGAGAACCCCCACCCCGCGTGCGGG

3.1.3 Recombinant Protein Production

The process of recombinant protein production involved PCR amplification of specific genes from *B. abortus* genomic DNA extracts, cloning amplified genes into pIVEX2.3d cloning vectors, and expressing recombinant proteins from cloned genes *in vitro*. Results from each stage of the protein production processes for each protein candidate selected during the bioinformatic analyses are presented in Table 5 and Table 6 for Chromosomes 1 and 2, respectively.

I. Amplification of ORFs

PCR amplification of selected ORFs using the designed oligonucleotide primers was successful for 92 out of 101 candidates. Candidates displayed in Table 5 and Table 6 as unsuccessfully amplified ORFs by the Phusion PCR assays were attempted multiple times with no change in the results. Candidates were selected for this study with PCR target gene amplification size limitations in mind. As a result, selected candidate ORFs ranged in size of 0.4 kb to 2.5 kb. The amplicons with the sizes in this range were detected under UV following agarose gel electrophoresis (see example in Figure 7). The PCR products with expected sizes were purified from the gels, quantified, and used for downstream cloning experiments.

II. Recombinant Plasmid Preparation

Successful results for recombinant plasmid isolation required multiple stages of confirmation with the final parameter requiring a high-quality alignment of sequenced inserts within recombinant plasmids with their respective reference genes. In order to obtain recombinant plasmids for sequencing and protein expression, it was first required to have transformed *E. coli* grown on selective media following the *in vivo* cloning experiments. Subsequently, colony cultures were prepared to propagate the recombinant plasmid containing a target ORF in *E. coli* and the presence of an inserted ORF within extracted plasmid DNA was

detected by colony PCR using Taq polymerase. Colony PCR with a recombinant plasmid derived from the pIVEX2.3d vector as the template required the T7 promoter and T7 terminator primer pair to amplify the target ORF inserted into the MCS. Colony PCR amplifications were confirmed to be successful if the expected size of ORF was found by agarose electrophoresis analysis (see example in Figure 8). When successful colony PCR results were obtained, recombinant plasmid DNA was extracted from respective candidate colony cultures and purified for outsourced Sanger sequencing from both directions with the T7 promoter and T7 terminator primers. Sequencing results were aligned with their reference genes in .FASTA format (see alignment examples of successfully expressed candidates in Figures 9 – 12 and examples in Figures 13 – 16 of candidates that did not express) to confirm that target ORFs had been cloned correctly and were ready for downstream protein expression testing. In the cases where all these requirements were not confirmed, candidates were not tested for expression. In total, 73 recombinant candidate plasmids were isolated and confirmed to have successfully cloned out of the 101 ORFs originally selected from the genome.

III. Recombinant Protein Expression

Protein expression was performed by using an *in vitro* transcription and translation kit employing *E. coli* expression machinery present in the cell-free lysate as well as T7 bacteriophage RNA polymerase. Expression success was evaluated by detecting 6x His tagged proteins through automated western blotting using the iBind system. iBind cards and separated preloaded wells exploited capillary action to run western blot antibody and wash reagents over the nitrocellulose membrane anchored proteins without user intervention. *In vitro* expressed proteins underwent a denaturing process to become unfolded and were separated by size through SDS-PAGE. Proteins were then transferred to nitrocellulose membranes through application of

electric currents to the polyacrylamide gels, allowing downstream detection of recombinant proteins immobilized on the nitrocellulose membranes using western blot assays (see example in Figure 17). A surprising number of protein candidates showed no detectable expression in western blotting following *in vitro* transcription and translation assays. Successful cloning of the candidate ORFs in frame with 6x His-tag coding sequences were confirmed by DNA sequencing. Twelve protein candidates that expressed consistently and were produced in suitable quantities advanced to downstream purification and immunoreactivity evaluation.

Table 5: Chromosome 1 Protein Production Results. Proteins displayed underwent production from genomic extracts of heat-killed *B. abortus* bv. 2 str. 86/8/59. Green coloured boxes represent condition success, red boxes represent condition failure. Genomic Amplification by Phusion polymerase PCR: "+" and "–" indicate a band of correct size was detected by UV fluorescence following PCR and electrophoresis or not, respectively. Recombinant Plasmid Isolation by Qiagen Minipreparation after colony PCR and alignments of candidate and reference sequences following Sanger Sequencing: "+" and "–" indicate a plasmid dually confirmed to have cloned successfully was isolated or not, respectively. *In Vitro* Protein Expression by RTS *E. coli* lysate: "+" and "–"indicate expressed protein His-tags were detected by western blot or not, respectively.

Candidate Accession Number	PCR Amplification	Recombinant Plasmid Isolation	*In Vitro* Protein Expression
AIJ92906	+	+	+
AIJ93147	+	+	+
AIJ91981	+	+	+
AIJ92065	+	+	+
AIJ92184	+	+	+
AIJ93502	+	+	–
AIJ92635	+	+	–
AIJ92714	+	+	–
AIJ92545	+	+	–
AIJ93692	+	+	–
AIJ92372	+	+	–
AIJ92062	+	+	–
AIJ93603	+	+	–
AIJ92115	+	+	–
AIJ92550	+	+	–
AIJ93181	+	+	–
AIJ93242	+	+	–
AIJ92270	+	+	–
AIJ91927	+	+	–
AIJ92208	+	+	–
AIJ92703	+	+	–
AIJ92314	+	+	–
AIJ93060	+	+	–
AIJ92581	+	+	–
AIJ92371	+	+	–
AIJ93725	+	+	–
AIJ92890	+	+	–
AIJ93662	+	+	–
AIJ92858	+	+	–

AIJ93619	+	+	–
AIJ93499	+	–	–
AIJ92061	+	–	–
AIJ93027	+	–	–
AIJ93481	+	–	–
AIJ93253	+	–	–
AIJ93047	+	–	–
AIJ93661	+	–	–
AIJ92992	+	–	–
AIJ92394	+	–	–
AIJ93639	+	–	–
AIJ91931	+	–	–
AIJ92261	+	–	–
AIJ93099	–	–	–
AIJ92748	–	–	–
AIJ93673	–	–	–
AIJ92038	–	–	–
AIJ91845	–	–	–
AIJ92709	–	–	–
AIJ92265	–	–	–
AIJ93597	–	–	–

Table 6: Chromosome 2 Protein Production Results. Proteins displayed underwent production from genomic extracts of heat-killed *B. abortus* bv. 2 str. 86/8/59. Green coloured boxes represent condition success, red boxes represent condition failure. Genomic Amplification by Phusion polymerase PCR: "+" and "–" indicate a band of correct size was detected by UV fluorescence following PCR and electrophoresis or not, respectively. Recombinant Plasmid Isolation by Qiagen Minipreparation after colony PCR and alignments of candidate and reference sequences following Sanger Sequencing: "+" and "–" indicate a plasmid dually confirmed to have cloned successfully was isolated or not, respectively. *In Vitro* Protein Expression by RTS *E. coli* lysate: "+" and "–"indicate expressed protein His-tags were detected by western blot or not, respectively.

Candidate Accession Number	PCR Amplification	Recombinant Plasmid Isolation	*In Vitro* Protein Expression
AIJ91039	+	+	+
AIJ90867	+	+	+
AIJ90882	+	+	+
AIJ90972	+	+	+
AIJ90995	+	+	+
AIJ91095	+	+	+
AIJ91201	+	+	+
AIJ91341	+	+	–
AIJ91442	+	+	–
AIJ91528	+	+	–
AIJ91673	+	+	–
AIJ90845	+	+	–
AIJ90810	+	+	–
AIJ90813	+	+	–
AIJ91347	+	+	–
AIJ90800	+	+	–
AIJ91026	+	+	–
AIJ90704	+	+	–
AIJ91445	+	+	–
AIJ91223	+	+	–
AIJ91379	+	+	–
AIJ91474	+	+	–
AIJ91202	+	+	–
AIJ91116	+	+	–
AIJ90955	+	+	–
AIJ90792	+	+	–
AIJ90806	+	+	–
AIJ90905	+	+	–
AIJ91123	+	+	–

AIJ91135	+	+	–
AIJ91153	+	+	–
AIJ91247	+	+	–
AIJ91268	+	+	–
AIJ91288	+	+	–
AIJ91360	+	+	–
AIJ91520	+	+	–
AIJ91621	+	+	–
AIJ91656	+	+	–
AIJ91664	+	+	–
AIJ91666	+	+	–
AIJ91738	+	+	–
AIJ91125	+	+	–
AIJ91259	+	+	–
AIJ91269	+	–	–
AIJ90888	+	–	–
AIJ91068	+	–	–
AIJ91663	+	–	–
AIJ91182	+	–	–
AIJ90956	+	–	–
AIJ91222	+	–	–
AIJ91734	–	–	–

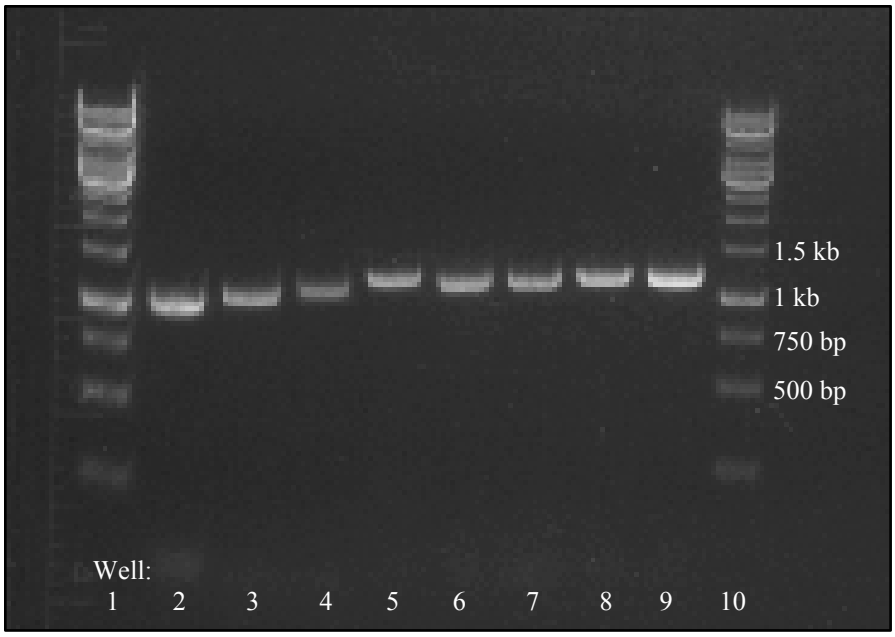


Figure 7: Phusion PCR 1% Agarose Gel Image. UV fluorescence of SYBR Safe DNA Gel Stain (Invitrogen, USA) captured for amplified genes. Well 1 and 10: 1 kb DNA Ladder (Thermo Scientific, USA); Well 2: AIJ92208; Well 3: AIJ93147; Well 4: AIJ92703; Well 5: AIJ92314; Well 6: AIJ93060; Well 7: AIJ92581; Well 8: AIJ91981; Well 9: AIJ92065.

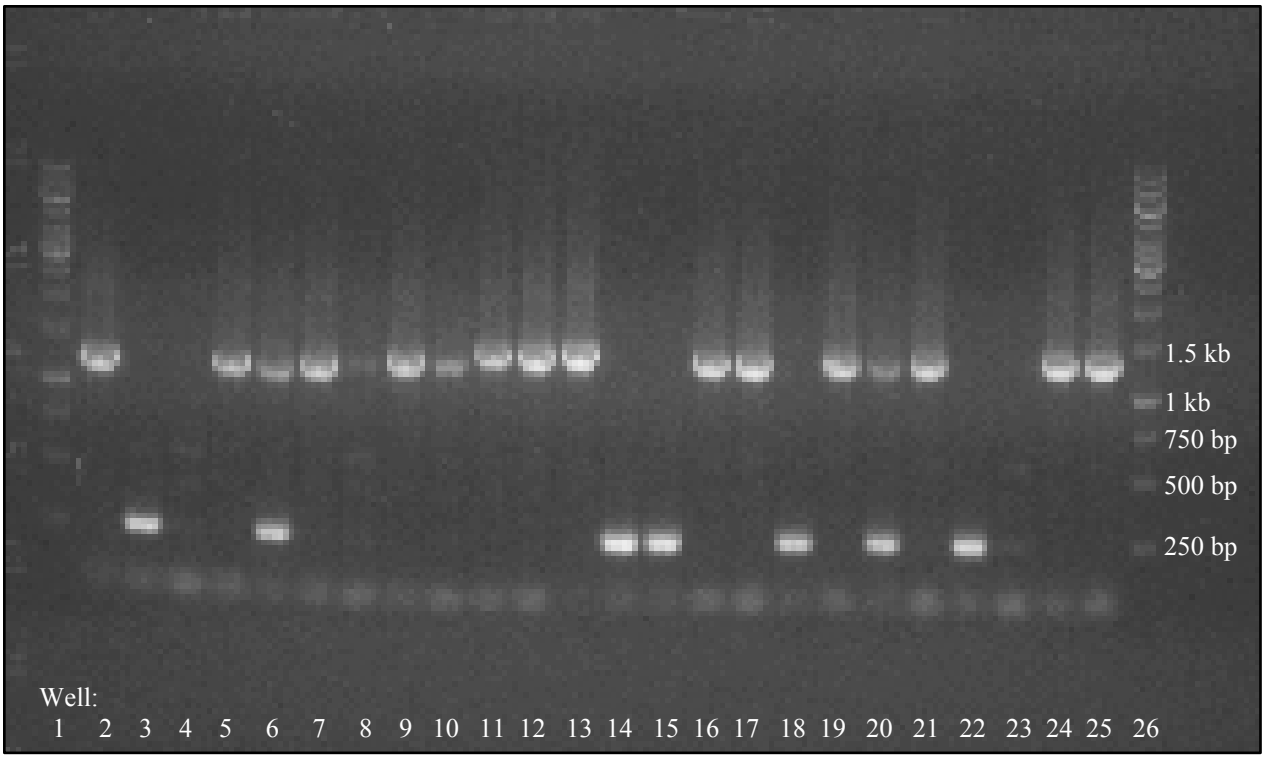


Figure 8: Taq Colony PCR 1% Agarose Gel Image. UV fluorescence of SYBR Safe DNA Gel Stain (Invitrogen, USA) captured for amplified T7 cloning regions of colony isolates containing recombinant pIVEX2.3d plasmids. Well 1 and 26: 1 kb DNA Ladder (Thermo Scientific, USA); Well 2: AIJ92208 colony 1; Well 3: AIJ92208 colony 2; Well 4: AIJ92208 colony 3; Well 5: AIJ93147 colony 1; Well 6: AIJ93147 colony 2; Well 7: AIJ93147 colony 3; Well 8: AIJ92703 colony 1; Well 9: AIJ92703 colony 2; Well 10: AIJ92703 colony 3; Well 11: AIJ92314 colony 1; Well 12: AIJ92314 colony 2; Well 13: AIJ92314 colony 3; Well 14: AIJ93060 colony 1; Well 15: AIJ93060 colony 2; Well 16: AIJ93060 colony 3; Well 17: AIJ92581 colony 1; Well 18: AIJ92581 colony 2; Well 19: AIJ92581 colony 3; Well 20: AIJ91981 colony 1; Well 21: AIJ91981 colony 2; Well 22: AIJ91981 colony 3; Well 23: AIJ92065 colony 1; Well 24: AIJ92065 colony 2; Well 25: AIJ92065 colony 3.

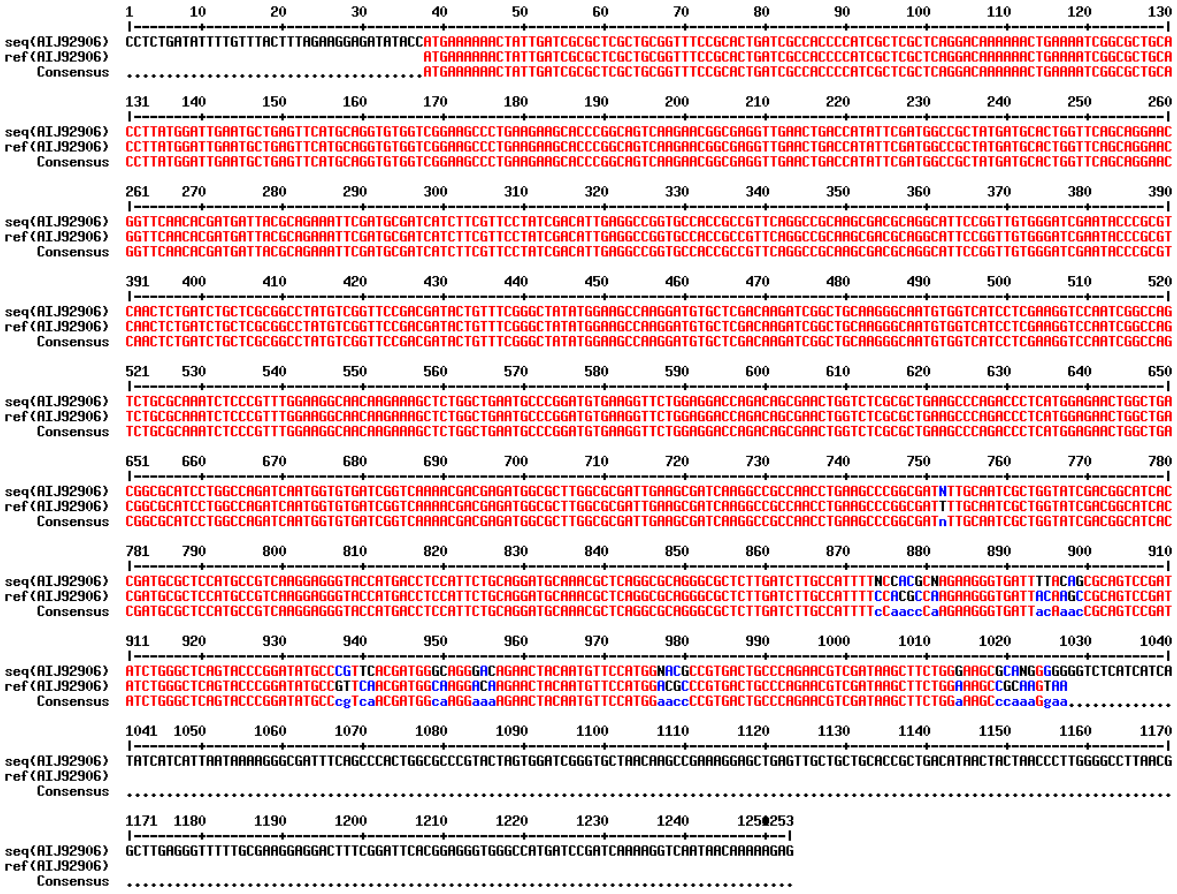


Figure 9: Alignment of AIJ92906 T7P with Reference Sequence. AIJ92906 is a chromosome 1 protein. Sanger sequence T7 promoter (T7P) direction displayed. Alignment has a gap penalty of 5 and no extension penalty. Red nucleotides indicate complete alignment, blue nucleotides indicate incomplete alignment, black nucleotides indicate no alignment.

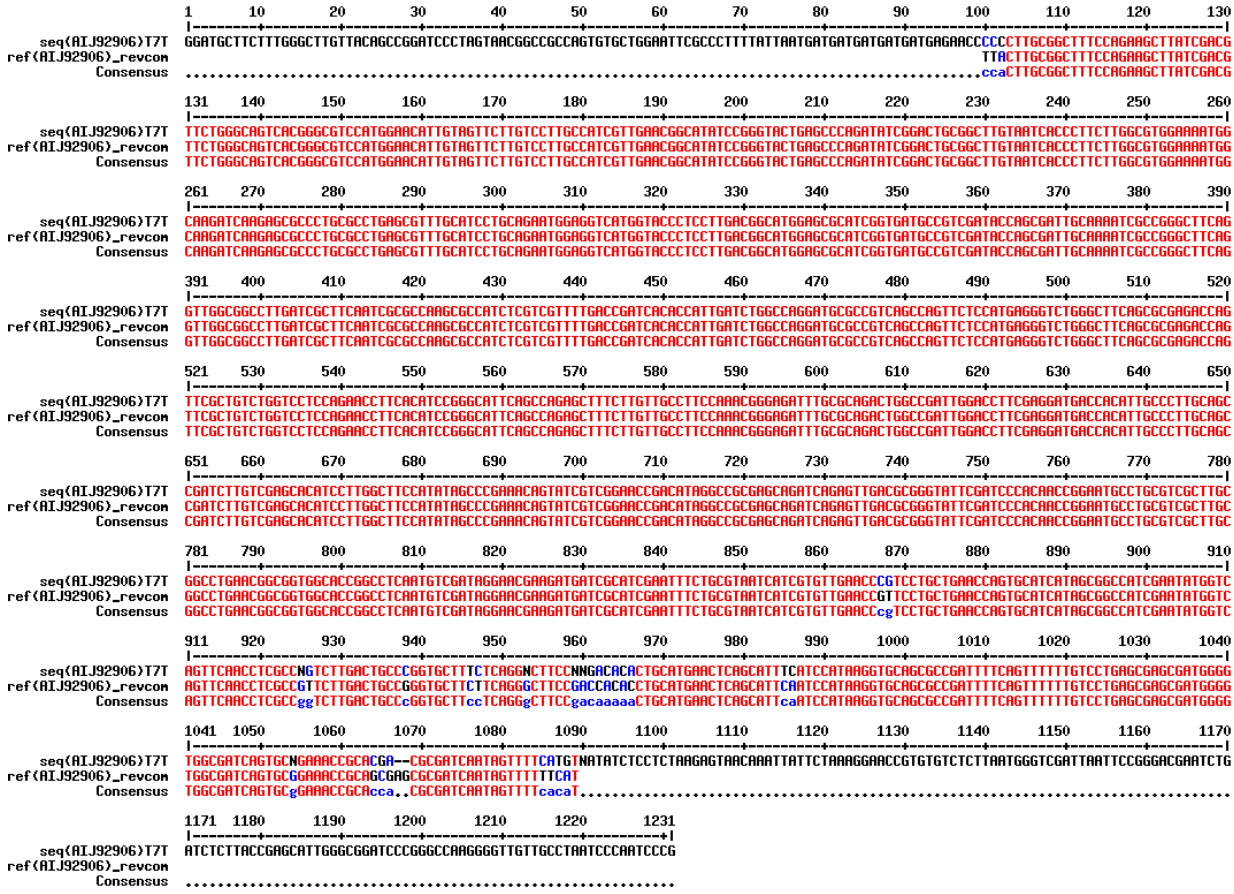


Figure 10: Alignment of AIJ92906 T7T with Reference Sequence. AIJ92906 is a chromosome 1 protein. Sanger sequence T7 terminator (T7T) direction displayed against the reference sequence in reverse complement. Alignment has a gap penalty of 5 and no extension penalty. Red nucleotides indicate complete alignment, blue nucleotides indicate incomplete alignment, black nucleotides indicate no alignment.

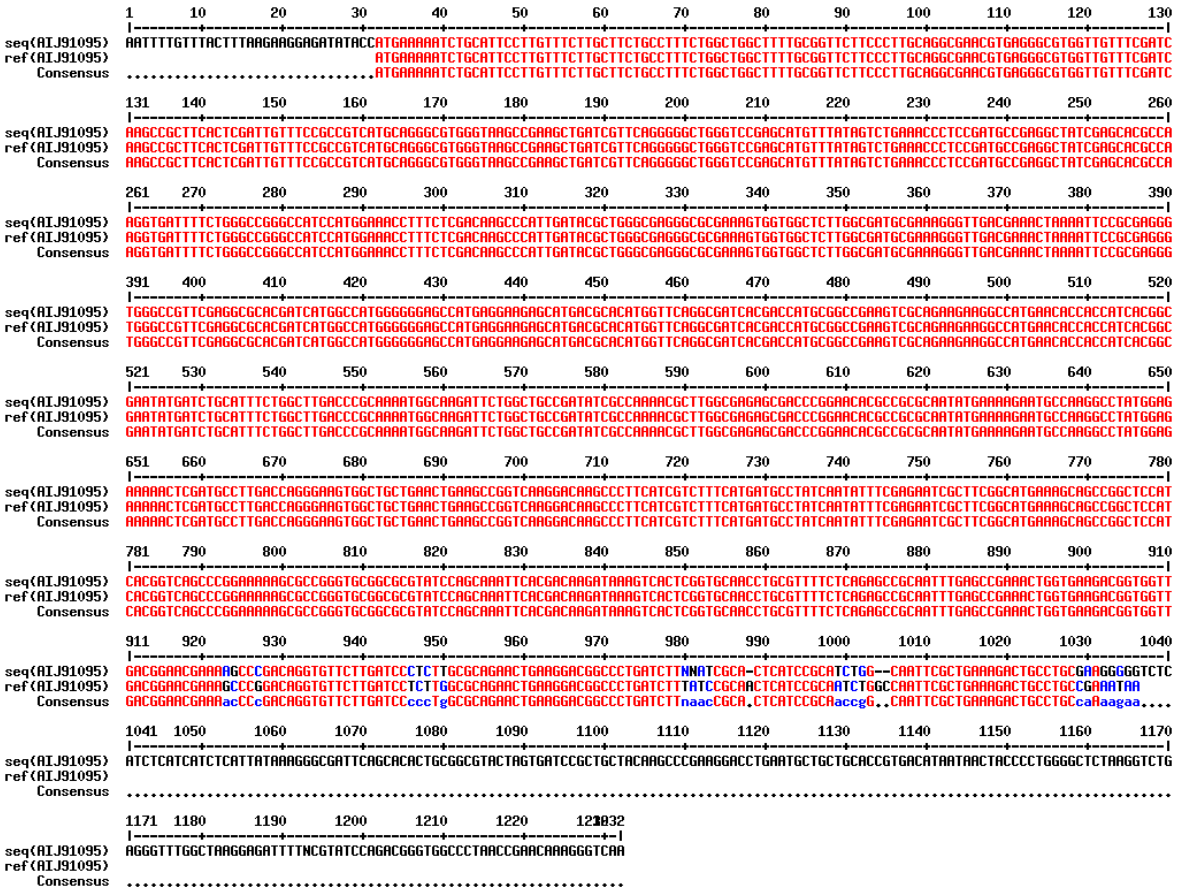


Figure 11: Alignment of AIJ91095 T7P with Reference Sequence. AIJ91095 is a chromosome 2 protein. Sanger sequence T7 promoter (T7P) direction displayed. Alignment has a gap penalty of 5 and no extension penalty. Red nucleotides indicate complete alignment, blue nucleotides indicate incomplete alignment, black nucleotides indicate no alignment.

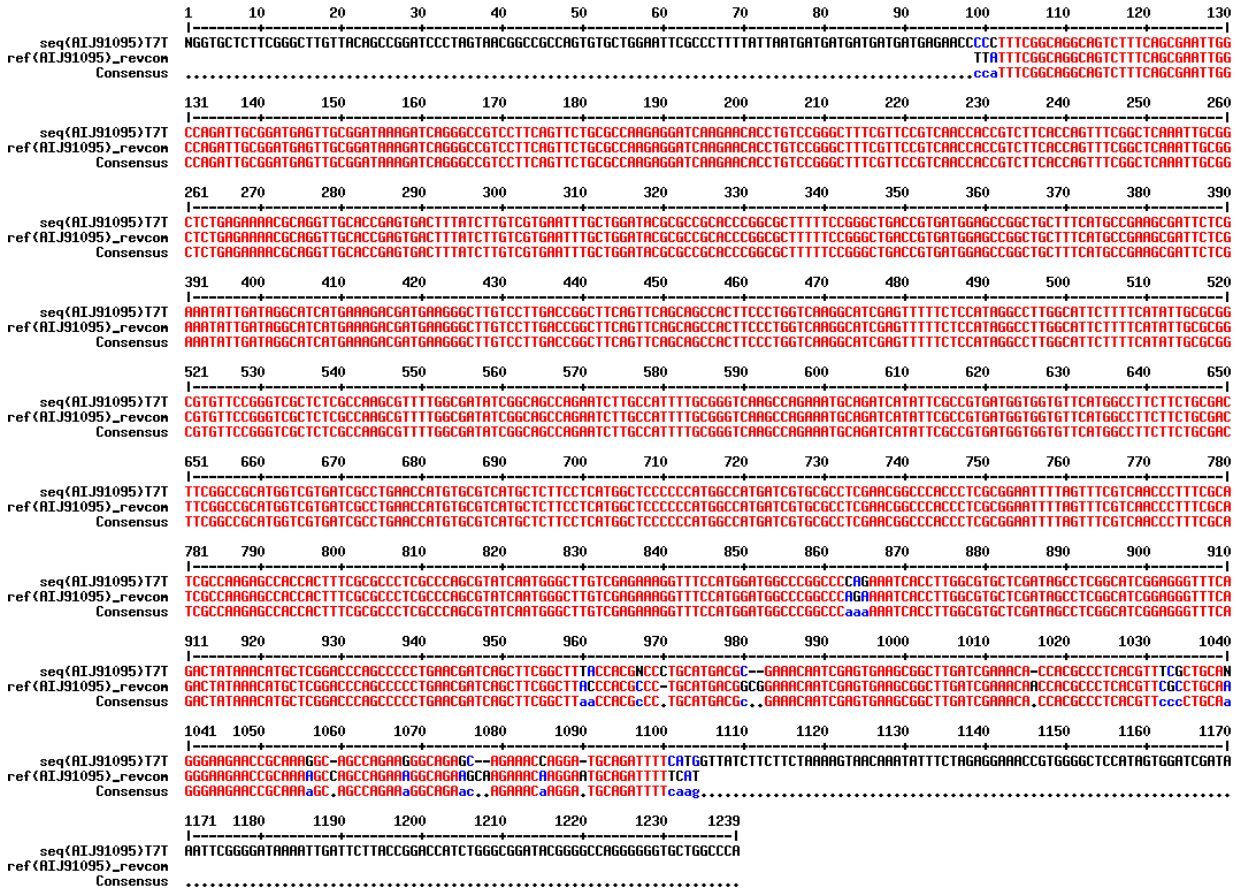


Figure 12: Alignment of AIJ91095 T7T with Reference Sequence. AIJ91095 is a chromosome 2 protein. Sanger sequence T7 terminator (T7T) direction displayed against the reference sequence in reverse complement. Alignment has a gap penalty of 5 and no extension penalty. Red nucleotides indicate complete alignment, blue nucleotides indicate incomplete alignment, black nucleotides indicate no alignment.

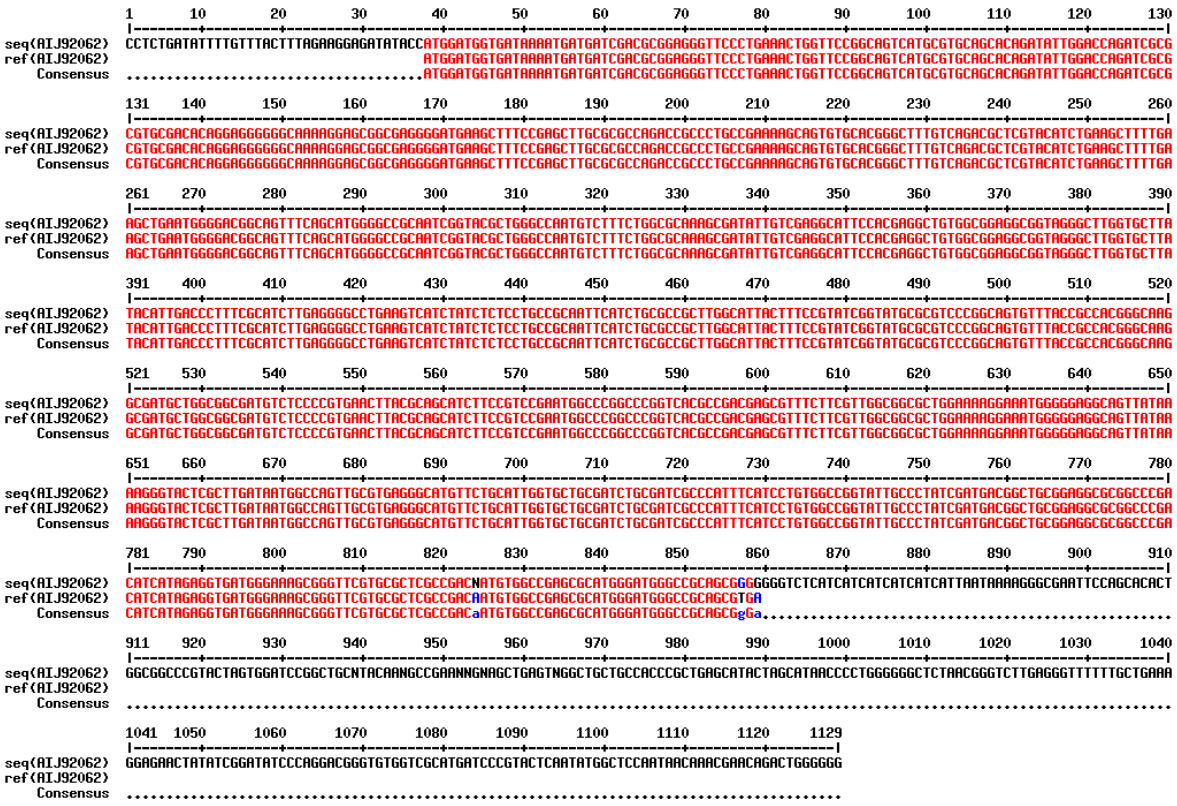


Figure 13: Alignment of AIJ92062 T7P with Reference Sequence. AIJ92062 is a chromosome 1 protein. Sanger sequence T7 promoter (T7P) direction displayed. Alignment has a gap penalty of 5 and no extension penalty. Red nucleotides indicate complete alignment, blue nucleotides indicate incomplete alignment, black nucleotides indicate no alignment.

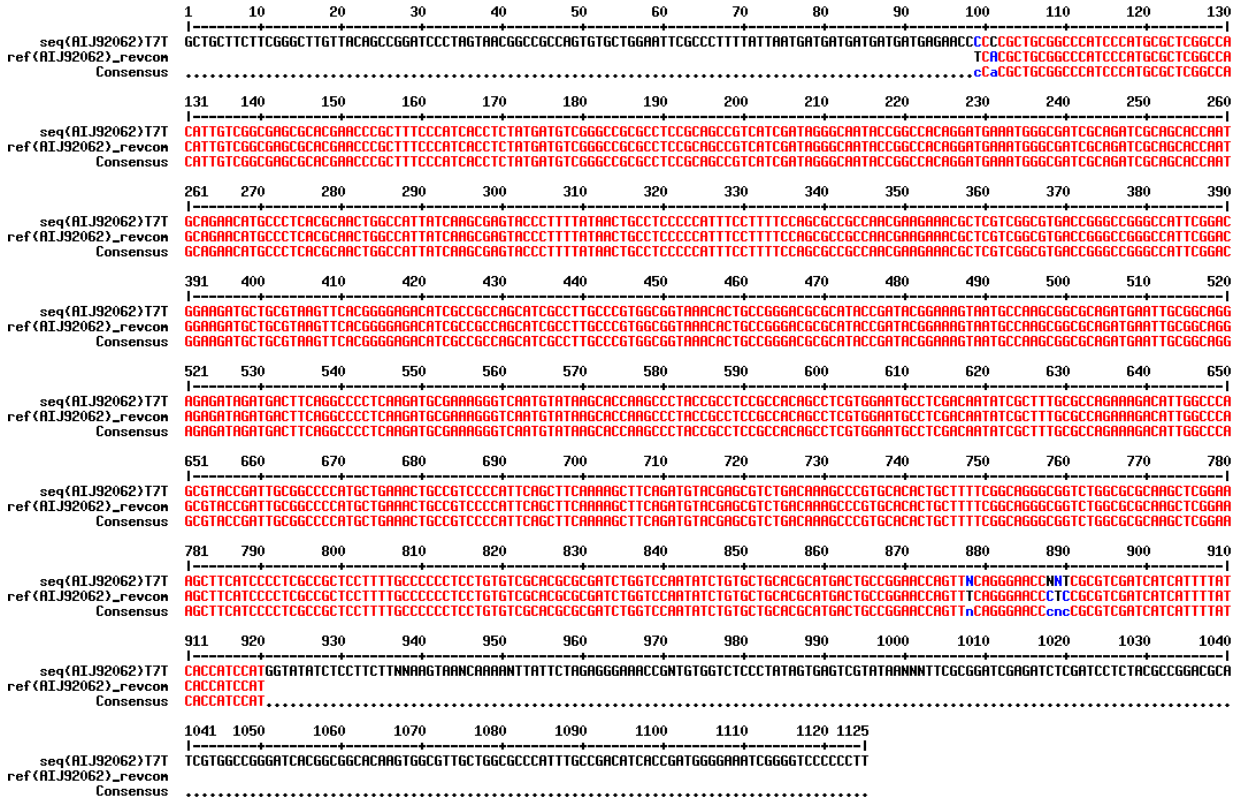


Figure 14: Alignment of AIJ92062 T7T with Reference Sequence. AIJ92062 is a chromosome 1 protein. Sanger sequence T7 terminator (T7T) direction displayed against the reference sequence in reverse complement. Alignment has a gap penalty of 5 and no extension penalty. Red nucleotides indicate complete alignment, blue nucleotides indicate incomplete alignment, black nucleotides indicate no alignment.

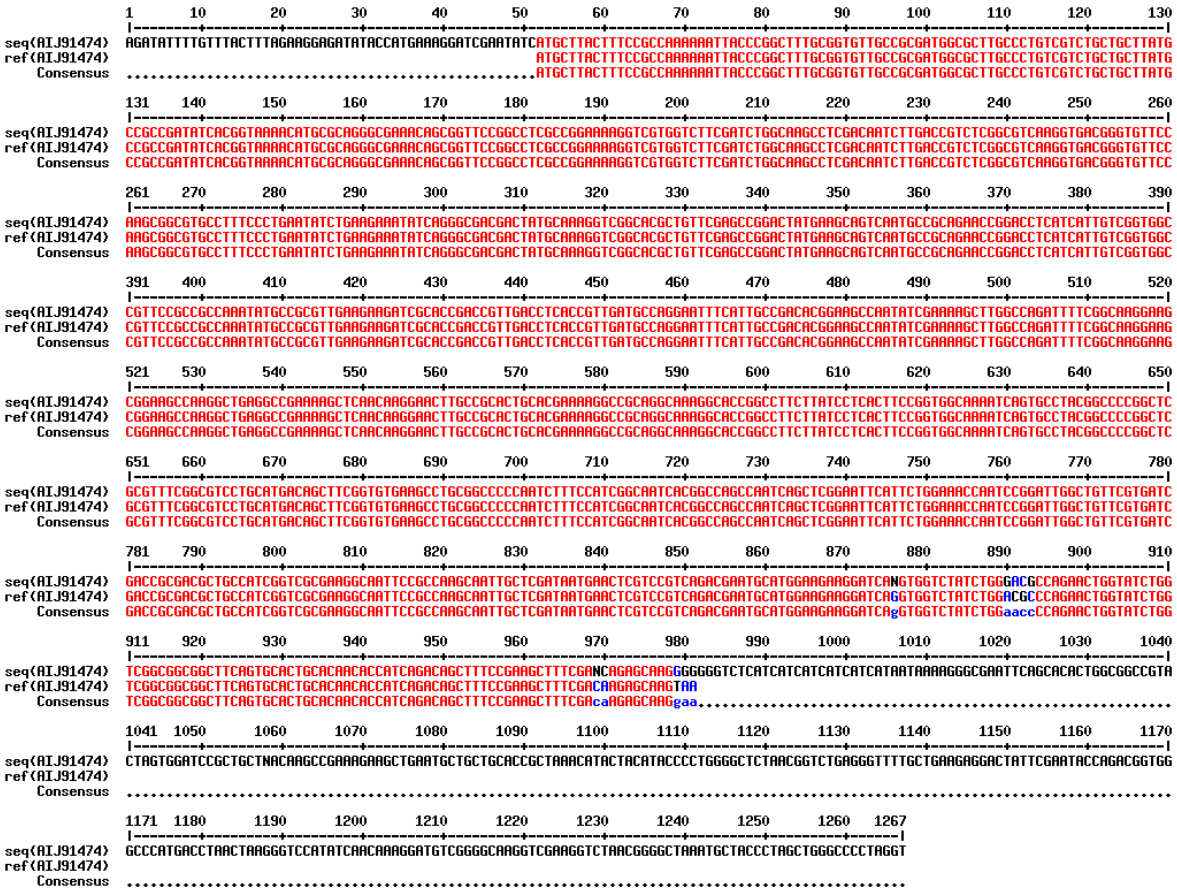


Figure 15: Alignment of AIJ91474 T7P with Reference Sequence. AIJ91474 is a chromosome 2 protein. Sanger sequence T7 promoter (T7P) direction displayed. Alignment has a gap penalty of 5 and no extension penalty. Red nucleotides indicate complete alignment, blue nucleotides indicate incomplete alignment, black nucleotides indicate no alignment.

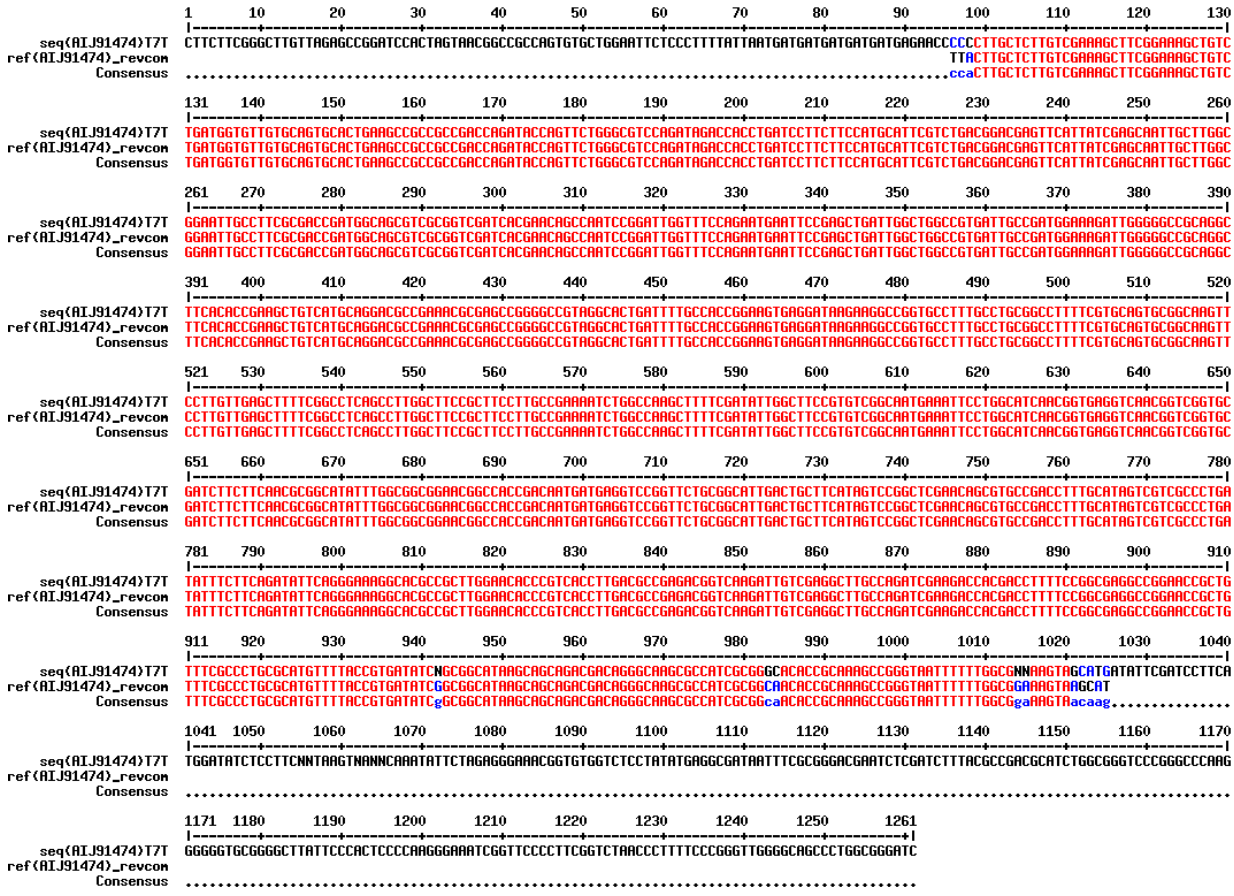


Figure 16: Alignment of AIJ91474 T7T with Reference Sequence. AIJ91474 is a chromosome 2 protein. Sanger sequence T7 terminator (T7T) direction displayed against the reference sequence in reverse complement. Alignment has a gap penalty of 5 and no extension penalty. Red nucleotides indicate complete alignment, blue nucleotides indicate incomplete alignment, black nucleotides indicate no alignment.

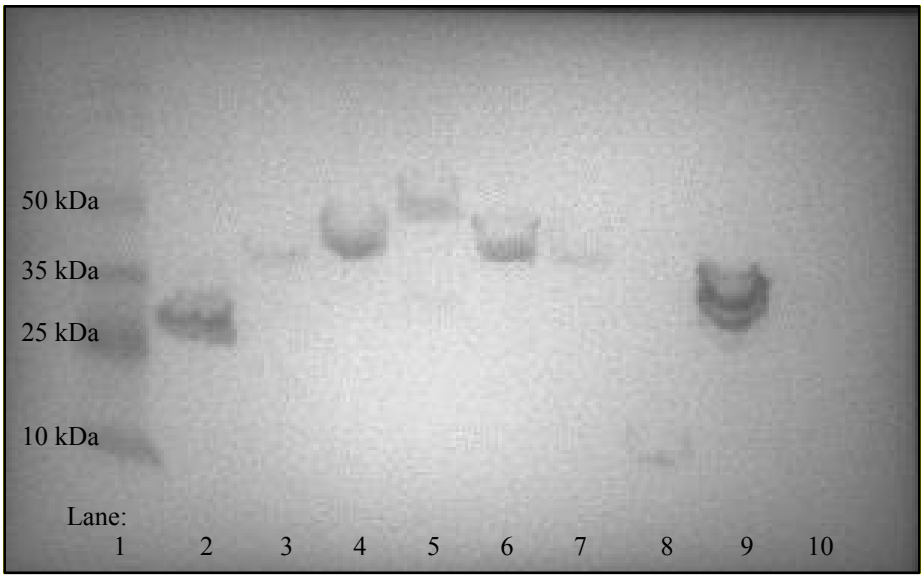


Figure 17: Western Blot Expression Confirmation. Expression reaction western blot on nitrocellulose. Membrane image captured following colour development. Lane 1: Prestained 10-250 kDa Protein Standards (BioRad, USA); Lane 2: Positive control (GFP); Lane 3: AIJ91095; Lane 4: AIJ91039; Lane 5: AIJ92184; Lane 6: AIJ91981; Lane 7: AIJ92065; Lane 8: AIJ93147; Lane 9: AIJ92906; Lane 10: AIJ93502.

3.1.4 Protein Purification

Ni-NTA agarose chromatography allowed for the purification of candidate proteins from the cell-free *E. coli* lysates through His-tag affinity for metal Ni^{2+} ions. Quantification of purified proteins is presented in Table 7. Following dilution in the Qubit Working Solution (WS), imidazole concentrations from Elution Buffer B used in purification were present over the recommended concentration by 5.15 mM in each protein sample and the protein free blank control. The blank control was used to determine the level of interference caused by imidazole at that concentration. As shown by the control quantification, the interference, while not ideal, was minimal. Using the maximum dilution factor recommended by the manufacturer of the purified protein samples in WS was attempted to bring the imidazole concentration into the recommended range, however, this yielded protein concentrations below the minimum detection threshold. Results from successful quantification testing provided confidence that samples contained purified proteins of sufficient quantities to be used in downstream screening for immunogenicity.

Table 7: Purified Protein Qubit Quantification Results. Concentrations of purified protein samples following expression. Results from all expressed candidates shown according to their NCBI accession numbers. Purified candidates suspended in Elution Buffer B from purification and diluted and Qubit Working Solution (WS). Blank Control contained protein free Elution Buffer B diluted in WS.

Protein Candidate Accession Numbers	Concentration Following Purification (ng/µL)*
AIJ92906	65.6
AIJ93147	86.4
AIJ92065	71.6
AIJ91981	84.0
AIJ92184	67.2
AIJ91039	73.2
AIJ91095	75.6
AIJ90972	70.8
AIJ90995	78.4
AIJ91201	61.6
AIJ90882	68.0
AIJ90867	66.4
Blank Control	21.4

*Imidazole was present over the recommended concentration by 5.15 mM in each sample.

3.1.5 Immunoreactivity Evaluation

Preparation of purified protein antigens in sufficient quantities allowed assessment of their immunogenicity by probing them with brucellosis positive and negative serum panels in dot blot assays. Table 8 presents screening results of reactivity between antibodies present in the bovine serum and the membrane-immobilized protein antigen candidates. Each nitrocellulose membrane array, containing all 12 purified protein candidates, was exposed to a separate diluted serum sample preparation in western blotting assays. All membranes also contained *B. abortus* LPS as the positive control and the recombinant CSFV E2 protein as the negative control. Sera and control antigens were sourced in-house from laboratory archives and conformational assessments were performed to demonstrate their reliability and suitability in preliminary reactivity testing prior to their use with candidates (See Appendix A1). Following iBind automated western blotting assays, chemiluminescence detections were performed to evaluate the immunogenicity of the protein antigen candidates (see Figures 18 and 19 for examples). iBind cards and preloaded wells exploited capillary action to run the western blots without user intervention, and proprietary iBind solution acted as both a blocking and washing buffer according to the manufacturer's specifications. Antigen spotting volumes and serum dilutions required for producing strong signals were determined experimentally (see Appendix A1). Positive and negative serum samples were shown to be reactive and non-reactive with the positive and negative controls, respectively, prior to testing with protein candidates. As seen in Table 8, none of the 12 protein candidates that were successfully produced and purified displayed any degree of immunoreactivity with the serum panels. Purified protein candidates were demonstrated to have been in sufficient quantity prior to immunoblotting and all control antigens consistently reacted as expected under the screening conditions employed. It is evident

that the positive sera from *B. abortus*-infected cattle did not contain antibodies against the 12 antigen candidates evaluated and, thus, these antigens did not play a role in the bovine immunogenic responses in the infected host cattle from which the positive sera were collected.

Table 8: Candidate Immunoreactivity Results. Chemiluminescence detection outcomes from antigen-serum interactions displayed. Green coloured boxes represent condition success, red boxes represent condition failure. Each purified protein candidate and control antigen were exposed to brucellosis positive and negative bovine serum panels to screen for immunoreactivity. Sera A – D are brucellosis positive. Sera E – H are brucellosis negative. "+" symbols indicate reactivity detected by chemiluminescence. "–" symbols indicate no reactivity detected by chemiluminescence.

		Positive Sera				**Negative Sera**			
	Sample	Serum A	Serum B	Serum C	Serum D	Serum E	Serum F	Serum G	Serum H
Positive Control	*Brucella* LPS	+	+	+	+	–	–	–	–
Negative Control	CSFV E2	–	–	–	–	–	–	–	–
Chromosome 1	AIJ92906	–	–	–	–	–	–	–	–
	AIJ93147	–	–	–	–	–	–	–	–
	AIJ91981	–	–	–	–	–	–	–	–
	AIJ92065	–	–	–	–	–	–	–	–
	AIJ92184	–	–	–	–	–	–	–	–
Chromosome 2	AIJ91039	–	–	–	–	–	–	–	–
	AIJ90867	–	–	–	–	–	–	–	–
	AIJ90882	–	–	–	–	–	–	–	–
	AIJ90972	–	–	–	–	–	–	–	–
	AIJ90995	–	–	–	–	–	–	–	–
	AIJ91095	–	–	–	–	–	–	–	–
	AIJ91201	–	–	–	–	–	–	–	–

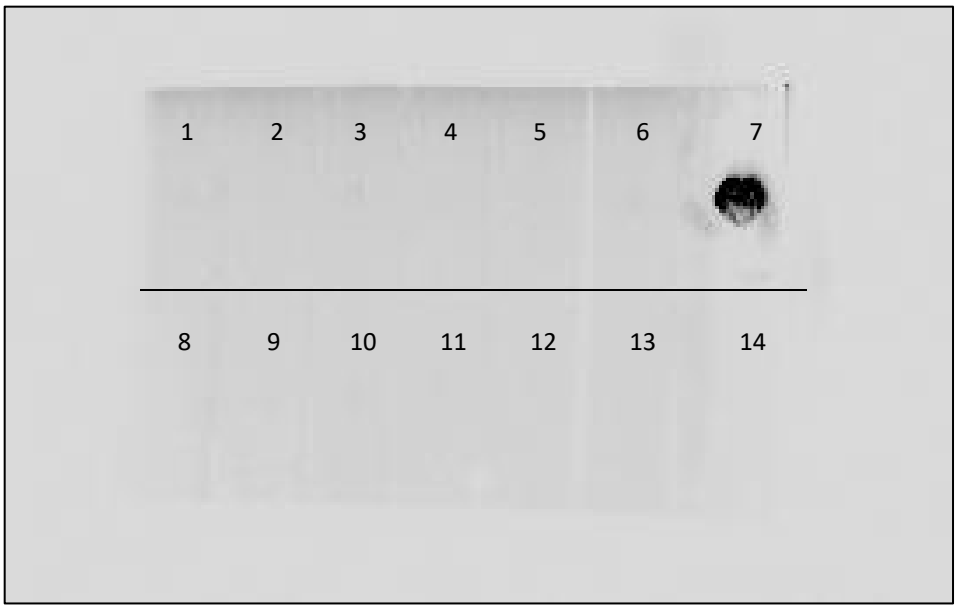


Figure 18: Serum A Candidate Immunoreactivity Assessment. Serum A is brucellosis positive. Image taken at the 3-minute development mark following initial exposure to ECL solution. Block 1: AIJ92906; Block 2: AIJ93147; Block 3: AIJ92065; Block 4: AIJ91981; Block 5: AIJ92184; Block 6: AIJ91039; Block 7: *Brucella* LPS; Block 8: AIJ91095; Block 9: AIJ90972; Block 10: AIJ90995; Block 11: AIJ91201; Block 12: AIJ90882; Block 13: AIJ90867; Block 14: CSFV E2 Protein.

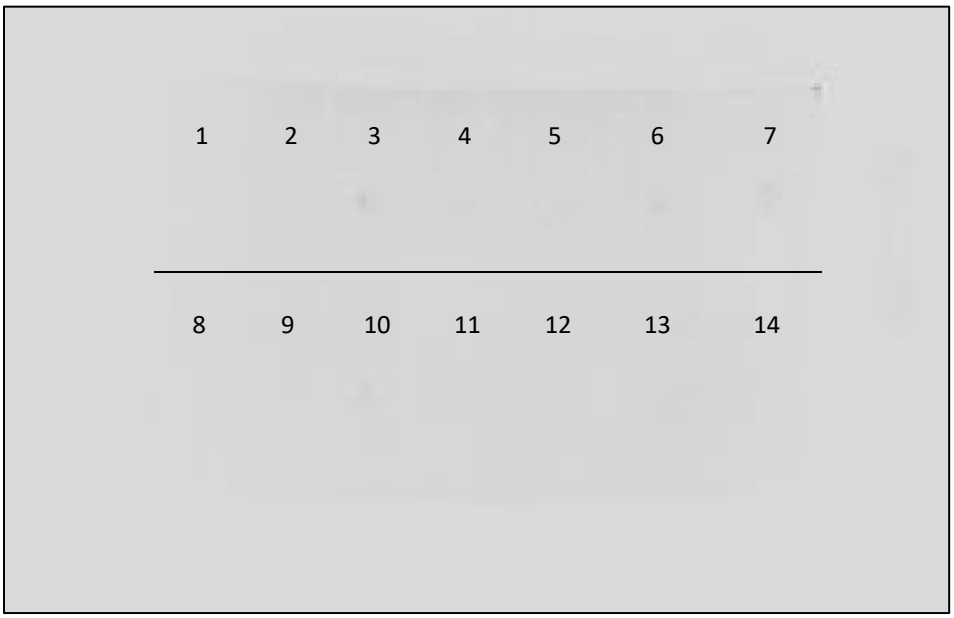


Figure 19: Serum E Candidate Immunoreactivity Assessment. Serum E is brucellosis negative. Image taken at the 3-minute development mark following initial exposure to ECL solution. Block 1: AIJ92906; Block 2: AIJ93147; Block 3: AIJ92065; Block 4: AIJ91981; Block 5: AIJ92184; Block 6: AIJ91039; Block 7: *Brucella* LPS; Block 8: AIJ91095; Block 9: AIJ90972; Block 10: AIJ90995; Block 11: AIJ91201; Block 12: AIJ90882; Block 13: AIJ90867; Block 14: CSFV E2 Protein.

4. Discussion

The major findings of this investigation were first and foremost that research into more candidates is needed. Additionally, it was demonstrated that the methods for amplification, purification, and cloning of candidate genes were effective following their selection using bioinformatics. The protein production and purification methods were also proven effective. Moreover, the expression system used was found to be unreliable for the recombinant candidate plasmids tested. However, an important finding was the proof-of-concept for the format of the immunoreactivity assays and chemiluminescent image capturing experiments developed that were consistently effective on the control antigens. This shows that the established workflow can be used to assess immunoreactivity of protein antigens to antibodies. These findings are significant because the extensive progress made for the technical development of antigen production and serological immunoreactivity testing will allow for streamlined testing of more candidates in the future. New knowledge generated by this research was mostly in advancing the technical methods involved in protein production and purification from protein extracts and the characterization of bovine serological immunoreactivity to those produced proteins. This research and other similar studies are of the utmost importance for the improvement of serodiagnostic testing specificity to allow for differentiation of infection between *Brucella* spp. and other Gram-negative bacterial pathogens (1, 46). While LPS antigens are highly sensitive when used in serodiagnostics, those assays lack specificity and multiple confirmatory tests are needed to confirm *Brucella* infection (5). This study helped work towards developing an improved serological test for brucellosis diagnostics by screening selected proteins encoded by the *B. abortus* genome for the discovery of novel antigens.

Multiple problems were solved from technical breakthroughs in the workflow such as the protein purification and quantification approaches used which allowed for a reduction in interference from residual antigenic material from the expression preparations. Additionally, the real-time chemiluminescence image capturing protocol was developed entirely as part of the research done for this thesis. The implication of this project is that this work laid wide-ranging foundations for the continued research into alternative antigens to LPS for use in serological testing for bovine brucellosis. The workflow established by this research project can be implemented for high-throughput screening of additional *B. abortus* proteins.

Interpretation of Results

For interpretation of the protein candidate selection results, the pursuit was to identify CE localized, extracellular, outer membrane, and signal peptide containing genes. Candidates between the size of 0.4 and 3 kb that had not been previously evaluated were selected for immunoreactivity screening with bovine serum. Proteins in that size range were ideal due to compatibility with downstream PCR and cloning assays without the need for modifications. The candidates' cellular localizations were predicted based on the PSORTb Bayesian network analysis. The Bayesian network was comprised of multiple localization prediction modules. Further genomic information – including protein accession numbers, non-redundant protein IDs, and genomic locus tags – was collected from NCBI.

To interpret protein production results in general terms, if bands were detected in genomic amplification PCR gel analyses, then a positive result was achieved at that stage for that candidate. If, following transformation and colony PCR, a high-fidelity alignment profile was observed between candidate recombinant plasmids and NCBI reference sequences, then a positive result was achieved for that candidate in the recombinant plasmid isolation stage.

Finally, if coloured bands were consistently detected in expressed protein immunodetection assays following western blotting, then a positive result was achieved for that candidate for expression.

When interpreting protein product results more specifically, amplification of selected candidate genes from genomic extracts was done through Phusion PCR because Phusion polymerase produces amplicons with high fidelity to their template sequences. High fidelity was required for high quality cloning and protein expression downstream. Successful PCR amplification involved a band of correct size being observed during detection of UV fluorescence in PCR gels (as shown in Figure 7). Failed amplification results were recorded when no bands of correct size were observed. A minimum of 3 independent attempts were made for all unamplified genes. Of note is the higher chance of amplification failure in candidate genes greater than 2 kb in length. Overall, the amplification phase was successful for the majority of the candidate pool.

Interpreting recombinant plasmid isolation results requires an understanding about experimental validation of the process as well as the controls used for verifying the results. Initially, transformed DH5α *E. coli* cells were grown on agar plates containing carbenicillin to select for colonies harbouring functional recombinant plasmids conferring antibiotic resistance. Cloning by transformation into pIVEX2.3d vectors produced recombinant proteins with added 6x His-tags on the C-terminus and involved using controls to validate reagent functionality prior to candidate work. Validation included separate positive controls to ensure DNA uptake and DNA ligation by competent *E. coli* cells *in vivo*. DNA uptake positive controls were solely undigested vector samples. Undigested pIVEX2.3d samples convey antibiotic resistance to carbenicillin present in the selective growth media, thus, when competent *E. coli* cells obtained

it, they were able to grow. Positive controls were solely purified NdeI digested vector samples. NdeI digestion linearized the pIVEX2.3d plasmids and left sticky ends at the restriction cut site. When digested vector DNA was taken up into competent cells, it was re-circularized through the ligation of those sticky ends, and conferred antibiotic resistance and allowed for growth on the selective media. Linearized pIVEX2.3d vectors with NdeI restriction cut site sticky ends do not confer antibiotic resistance without uptake and plasmid recircularization by *in vivo* ligation. All positive controls were diluted 100-fold prior to plate culturing to allow for single colony growth rather than bacterial lawns. Negative controls were composed of digested vector amplicons with MCS blunt ends but were absent of any candidate amplicon samples. Digested vector amplicons that contained blunt ends at the MCS, which were homologous to the cloning sequences added to candidate gene amplicons, were ready for homologous recombination. When candidate amplicons were not present, vector blunt ends were unable to be recircularized through ligation, resulting in a failure of the respective competent *E. coli* cells to grow on the selective media. This allowed for an effective negative control. Following positive and negative control verification of the materials required for cloning by transformation assays, cloning was attempted for each amplified candidate. When purified candidate gene amplicons and prepared blunt end vectors were included together with the heat-shocked host cells, uptake and transformation could occur and candidate amplicons could clone into pIVEX2.3d plasmid vectors through homologous recombination *in vivo*. They could then be isolated from transformed CFUs growing on the selective media. Following that, colony PCR assays were able to provide crude confirmation of cloning success through bp length analysis of candidate amplicon inserts present in the MCS of pIVEX2.3d plasmid vectors. Colony PCR assays were performed using Taq polymerase since plasmid T7 region amplicon sequences were not required

to have high fidelity to their corresponding insert sequences. When colony samples were found to have inserts of correct size in PCR gel analyses, broth culture glycerol stocks were preserved and stored at –80°C. In cases where none of the initial 3 colonies were found to have correct insert sizes, additional colonies were selected and broths were prepared and screened. In cases where the screening of many colonies yielded no detection of successfully cloned plasmids, transformation was reattempted. For candidates that did not result in colony growth on selective media following transformation, or yielded colonies that did not contain expected recombinant plasmid sizes, transformation was reattempted to a maximum of 3 attempts. The most common case of failure was the lack of any colonies growing on selective media following transformation. Another common occurrence was the detection of empty plasmid vectors in colony PCR which had either recombined with themselves or stray DNA fragments, which allowed for growth on the selective media. Such samples were, likewise, not extracted or sequenced. Another area of failure was an incorrect size band detected, although this was rare. All candidates with expected colony PCR results that were sequenced, and subsequently tested for expression, had strong alignments with their reference genes. Candidates without detectable plasmid inserts of correct size, following multiple attempts, were marked as unsuccessful and were not isolated for sequencing and downstream evaluation. The creation of colony broths, while requiring an extra day of incubation, was beneficial because it allowed for the availability of ample volumes of culture for long term storage and downstream assays.

Interpretation of the cloning results required an understanding that Sanger sequencing is the best method for shorter length sequences such as the individual candidate genes cloned into the T7 region of pIVEX2.3d plasmid vectors. The minimum concentration of recombinant plasmid DNA samples required for Sanger sequencing was 50 ng/μL. In the results, it can be

seen that Sanger sequencing becomes less accurate at the ends of the reads due to target gene length limitations of the polymerase enzymes utilized. Therefore, sequencing from both directions was necessary, which further displayed the high-quality cloning success of 73 candidates.

Protein expression results must be interpreted by reviewing details of the expression system utilized. That expression system is capable of producing up to 20 μg of protein per 50 μL reaction according to the manufacturer. Application of the expression system for *in vitro* transcription and translation was beneficial insofar that it was a resourcefully inexpensive method and encompassed a relatively simple approach for its integration into standard molecular experimentation frameworks. In chromosome 1, 25 out of 30 candidates that cloned successfully failed to show detectable signals in western blotting assays. In chromosome 2, 36 out of 43 candidates also failed. A total of 12 proteins had detectable bound antibody signals and were able to be consistently expressed and produced in suitable quantities for downstream purification and immunoreactivity evaluation. All candidates that failed were tested multiple times and, in every case, GFP positive expression controls consistently gave strong signals during western blotting. All candidate recombinant plasmids that were tested in expression displayed high-quality cloning success and near perfect alignment with their respective reference sequences. Due to this extraordinary level of confidence in cloning success and the reliable effectiveness of positive controls, it was determined that unsuccessful cloning and expression system reaction errors were not factors in cases of candidate expression failure. For candidates that dependably expressed, Ni-NTA chromatography was employed and is capable of purifying >95% of contaminants present in lysate solutions with a binding capacity of up to 50 mg/ml and a yield of 100 μg – 100 mg according to the manufacturer. Ni-NTA chromatography functions through His-tag affinity

binding and elution using imidazole as a His-tag binding competitor for Ni-NTA. Protein candidates required purification prior to immunoreactivity testing due to the presence of residual *E. coli* protein and LPS contaminants in expression lysate solutions. Residual *E. coli* contaminants in candidate samples would have caused interference in immunoreactivity detection downstream. All cattle are exposed to environmental *E. coli*, which results in correspondingly reactive antibodies present in their sera. Similarly, environmental *E. coli* that cattle are exposed to paired with *E. coli protein* antigens present in expression reaction lysate solutions is the cause for such interference. To avoid this, impurities were removed through Ni-NTA chromatography which only binds to proteins that contain 6x His-tags to its residues.

For interpreting the purified protein quantification measurement results, it is of note that the purification elution buffer contained concentrations of imidazole 5.15 mM above the manufacturer's recommendations for the quantification assays. This potentially caused interference with fluorometric readings. Following purification, multiple quantification methods were investigated to establish which would be the most sensitive with the smallest sample volume required. Invitrogen's Qubit method was found to be the most efficient option for that. Selection of the Qubit approach was based on its sensitivity, reliability, and the automated aspects of the quantification procedure. The potential for quantification value inflation due to imidazole interference was determined to be minimal through use of a blank control that contained protein free elution buffer only. Testing purified protein solutions that were prepared with a greater dilution factor was attempted with the goal being to bring imidazole concentrations within the recommended limits. However, that level of dilution resulted in protein candidate concentrations falling below minimum detection thresholds and a failure to obtain any quantification values. Quantification of unpurified expression reactions would have been,

likewise, uninformative due to the many contaminants present in expression reaction solutions including *E. coli* transcription and translation machinery proteins. Alternative quantification measures considered included the Bradford dye-binding method and the Pierce BCA Protein Assay. The Bradford method required greater protein sample dilutions in colorimetric dye reagents than Qubit protocols and its minimum detection threshold was also less sensitive. Moreover, imidazole was additionally listed as a contaminant with potential to cause spectrophotometric interference in this method as well. Pierce quantification assays were impractical due to their requirement of large volumes of purified protein samples greater than each entire expression reaction solution. Additionally, in Pierce assays, imidazole was also listed as an interfering contaminant. While protein expression success rates were low overall, expression yields of successful candidates were likewise low. Due to this, in any of the aforementioned quantification methods, dilution of purified protein samples in quantification reagents beyond a small degree resulted in no proteins being detected at all as the proteins were diluted below minimum detection thresholds. Boosting concentrations by combining many expression reactions was possible but costly, as each expression kit contained reagents for only 24 expression reactions. In general, 10 – 100 ng of protein is the common amount required for western blotting assays and, based on this, the obtained quantification results were determined to be acceptable. Correspondingly, any remaining concentration uncertainty was mitigated through the use of increased spotting volumes in downstream immunoreactivity testing. Furthermore, as related to spotting volumes, minimum anticipated concentrations of purified protein solutions were required to be at least 30 ng/μL, a value which all purified candidates were quantified well above of.

The main advantage of using iBind in western immunoreactivity assays is that it requires lower volumes of antibody than traditional western configurations. This was especially advantageous due to brucellosis positive sera being exceptionally difficult to obtain and limited sample volume and supply availability from the CFIA biological archives of past outbreak isolates. Conventional western blotting requires 5x more primary antibody solution than iBind. Considering the 1/10 serum dilution required for strong positive control reaction signals, iBind provided a practical solution to performing as many tests as possible with the limited volume of sera available.

Validation of materials involved in immunogenic evaluation assays that had been sourced in-house was necessary prior to their use in candidate screening. This was done to minimize downstream troubleshooting. All sera and control antigens were sourced in-house from CFIA and Agriculture Canada biological archives of past outbreak isolates. Archived samples used in testing were stored at –80°C prior to retrieval. CSFV E2 proteins were used as negative controls because they are immunoreactive antigens and are unrelated to common cattle pathogenic exposure, bovine brucellosis, or *Brucella* spp. Correspondingly, any ECL reaction signal detected from respective E2 control dot blot sectors would indicate reaction condition inaccuracies and, consequently, the invalidation of any results from that protein array. Since the role of E2 proteins was to not elicit any reaction in immunoreactivity arrays, approaches alternative to basic serum reactivity assays were employed in order to confirm the viability of the available E2 protein samples (see Appendix A1). Validation of *Brucella* LPS, sourced in-house, was comprised of preliminary dot blot reactivity analysis with the positive serum panel followed by ECL. Serum panels were validated through preliminary dot blot reactivity assessments with positive and negative control antigens and ECL. Positive sera were assessed by their reaction to

directly spotted LPS positive controls on nitrocellulose membrane strips. For positive sera, performance was expected to encompass strong signals in ECL on positive control spots and no reaction from negative control spots. Negative sera were expected to show no reaction with either of the controls when assessed by ECL.

Candidate immunoreactivity results are interpreted based on their testing of different sera for each array. All arrays contained all expressed candidates, validated *Brucella* LPS positive control antigens, and CSFV E2 protein negative control antigens. Due to the standard volumetric choice for antigen spotting, greater amounts of candidate and controls than commonly used in western blotting may have been present on membrane arrays. However, use of such volumes might have allowed for the mitigation of any inflation in quantification measurements potentially caused by fluorometric interference from candidate and control diluent reagents.

Each serum panel was composed of 4 high quality samples, validated against control antigens through western dot blots and ECL reactivity analyses. Results were obtained for ECL peroxidase reactions in real-time allowing for recurring image capture of any positive immunoreactivity signals as they occurred. Image capture on the Gel Documentation System was programed to occur every 30 seconds for 3 minutes. After 3 minutes, background noise would begin to interfere with image clarity and at less than 3 minutes, positive control signals were not at maximum intensity. Any positive candidate reactions were anticipated to progress at a similar rate to the positive controls. Thus, a lack of signal from any dot blot spot by the 3-minute cut off time was considered negative since positive control signals were at their strongest by that point. Results from candidate immunoreactivity screening showed no positive reaction signals from any brucellosis negative sera with candidates or controls. Such outcomes from brucellosis negative sera were ideal as they indicated that the candidates tested were not common bovine

antigens – to which antibodies would be produced due to natural environmental exposure – and that the source specimens of the negative sera were not previously exposed to *Brucella*. Results from positive sera arrays showed consistent reactivity with the *Brucella* LPS positive controls, though no reaction with expressed protein candidates. Despite validation of immunoreactivity evaluation materials and assays prior to candidate screening, there were no positive reaction signals detected from any of the protein candidates with any of the positive serum samples. Overall, these results indicated that more candidates need to be produced and screened in order to contribute to the discovery of *B. abortus* proteins with serodiagnostic potential.

Due to the lack of positive immunoreactivity results, there is limited commentary that can be given regarding comparison of the results in this thesis to those of others. However, the previous *B. abortus* project which evaluated a separate protein cohort of 100 candidates, led to the identification of 10 protein antigens with the diagnostic potential (1). Another project in our research group that worked to identify immunoreactive *Mycobacterium bovis* proteins identified 2 protein candidates which showed consistent immunoreactivity with *M. bovis* positive bovine serum out of 159 proteins tested which gave an ~1.25% success rate (114). In 2005, Davies *et al.* carried out a study aiming to identify immunoreactive proteins from vaccinia virus using a protocol similar to the one described in the methods section previously. Davies *et al.* reported that 13 out of 185 evaluated protein candidates exhibited immunoreactivity with antibodies in sera from vaccinia virus-immunized humans. They also reported a 93% success rate for *in vivo* cloning and a 96% rate for PCR amplification success (92). Although the reported success rates for the identification of immunoreactive proteins from the vaccinia virus and *M. bovis* studies are higher than the one experienced in this thesis, they are still considered low. This low rate suggests that many *B. abortus* protein candidates must be screened in order to identify a few

antigens that may have potential for use in serodiagnostic tests. In contrast to the results of this study, other studies within our research group revealed a 50 – 90% success rate for candidate expression with the type of *in vitro* protein transcription and translation kit used in this project (1, 114). The expression success rates experienced for candidates tested in this project was under 12%. This is inconsistent with the manufacturers specifications as well as within the research group and scientific literature. The cause of so few candidates expressing is unknown as they were confirmed to have all cloned with near perfect fidelity to their respective reference sequences. Positive control expression reactions also always succeeded. Possible explanations for the consistent candidate expression failure may be due to protein insolubility or potentially since the proteins were predominantly periplasmic, that may have somehow caused an incompatibility with the *E. coli* expression machinery utilized by the expression kit.

Future Directions and Final Conclusions

As a whole, this project established a screening protocol for protein antigen identification and evaluation for serological reactivity using emerging technologies such as machine learning and automation. More work building on this project's methods and results is needed in the field to continue approaching the issue of improving bovine brucellosis serodiagnostics using modern scientific resources.

For candidate selection, more *Brucella* genomes can be analyzed from additional strains and species to expand and diversify the library of proteins that can be evaluated for their serodiagnostic potential. In protein production, several key areas of difficulty were unable to be addressed within the scope and time limitation of this project. These mainly included additional studies into alternative methods of molecular protein formulation for the many candidates that failed at the various stages of production. For candidates that failed to amplify during Phusion

PCR, chimerization or fragmentation modifications of protein coding genes could potentially lead to successful PCR amplification. However, downstream protein reconstruction of chimerized or fragmented antigens would be required prior to immunoreactivity evaluation. Furthermore, future applications of alternative cloning and expression methods may help address protein production issues. In cases of unsuccessful *in vitro* expression, the plasmid vector pIVEX2.4d could be used to produce recombinant proteins with N-terminal 6x His tags. This would require different PCR primer modifications, though a change in His-tag orientation may have a positive effect on cloning and expression for candidates that failed to do so in pIVEX2.3d vectors. Additionally, recombinant protein expression could be performed in later studies using an *in vitro* mammalian transcription and translation system with pT7CFE1 mammalian vectors producing recombinant proteins with C-terminal His-tags. Large scale culture based *in vivo* expression methods could also be implemented and may have higher success rates for candidates that failed to express *in vitro* in this study. However, such configurations generally require litres of liquid broth media for each candidate and when large numbers of candidates are being evaluated, production and management of many high-volume broth cultures could become challenging. In future studies, alternative protein purification and elution methods could also be explored to avoid quantification interference issues experienced in this study from the Ni-NTA eluent imidazole.

Regarding candidate screening for serological reactivity, several considerations for future directions could be explored. Firstly, more candidates could be produced and tested against positive serum panels to continue the search for immunoreactive proteins. This includes screening of all candidates that failed to amplify, clone, and express in this project once troubleshooting allows for their reliable production. Secondly, the serum panels themselves can

be expanded to provide more comprehensive reactivity profiles for any future candidates found to be reactive – this would be dependent, however, on the availability of quality brucellosis positive serum samples. Conversely, the use of commercial sera and controls instead of archived isolates from long term storage may have an impact on future serological results. Another avenue for examination in serological assays could be the use of a protein positive control. This would allow for a more direct reactivity comparison between a known immunoreactive protein antigen and any newly discovered immunoreactive proteins. Furthermore, upon discovery of novel reactive candidates, supplementary fine tuning of detection assays could be performed. This could include determination of minimum concentration values and volumes required from proteins and sera for reliable strong positive signals. Additionally, once a candidate displays immunoreactivity, it would need to be cloned into a new vector and produced in large quantities *in vivo* followed by immunoreactivity validation through rigorous ELISA testing against large panels of positive and negative sera. Following that, statistical analysis would be required to confirm significance and for determination of minimum thresholds for diagnostic sensitivity, as well as strain and species reaction specificity. Candidates validated through such testing would have potential for use in high throughput serodiagnostic field screening of *B. abortus* exposure and infection in domestic livestock. Possible alternative approaches that could also have been implemented into the project and may have potentially improved the production, detection, and discovery of immunogenic *B. abortus* antigens include using ELISAs in the place of the immune dot blots for evaluating the reactivity of protein candidates against brucellosis positive bovine sera. Different *in vitro* transcription and translation systems could have been utilized as well. There are various commercial *in vitro* expression kits available, such as one from Qiagen, that vary in terms of the components used to achieve *in vitro* expression as well as the conditions

required by the assays. There are many commercial options for different plasmid vectors as well that could have been assessed and utilized for *in vivo* cloning by homologous recombination. These options may have had resulted in a higher success rate for the *in vitro* expression or could be attempted to expresses those candidates that failed to do so in the recombinant constructs derived from the pIVEX2.3d plasmid vectors used in this study. Mammalian cell-free expression systems and vectors are also available for *in vitro* protein expression. Another feasible option is performing protein expression *in vivo* in *E. coli* instead of using the *in vitro* cell-free expression format.

Not only was the fundamental goal of this project to find immunoreactive proteins, but to also find those that illicit the most powerful immune responses within immunoreactive protein populations. Since no immunoreactive proteins were identified in this project, addressing these goals could be attempted in future studies since finding such candidates could have significant positive impacts on brucellosis serodiagnostics. Integration of these potential solutions and strategies to address the issues faced by the project was not possible due to the pursuit of obtaining serological evaluation results for the successfully expressed candidates. Nevertheless, this workflow can be used in the future to evaluate more protein candidates, other species and genera, as well as for other pathogen-based diseases in general, and may contribute towards improving the overall field of serodiagnostic pathogen testing.

This project worked towards addressing the gap in the literature for *B. abortus* bv. 2 str. 86/8/59 regarding the immunological evaluation of its proteins. Results from this project are preliminary but promising for contribution to the field of brucellosis serodiagnostics through the development of an optimized workflow for identification of immunoreactive candidates. New technologies implemented by this study came with their own unique challenges but ancillary

antigen library building and expansion are needed alongside continued optimization of immunoreactivity evaluation techniques. The project's hypothesis was not disproven by the results from each objective; however, additional protein evaluation is necessary and further work is required to address the issue of coming to a single test for brucellosis serodiagnostics. In conclusion, this study aimed to contribute to the discovery of protein antigens in the *B. abortus* bv. 2 str. 86/8/59 genome and the evaluation of their serodiagnostic potential through implementation of bioinformatics, molecular protein formulation, and serological reactivity testing.

5. Appendices

A1: Serological Reagent Validation Prior to Use in Candidate Reactivity Testing

A1.1 CSFV E2 Protein Sample Confirmatory Assays

Coomassie Blue Protein Staining. Following SDS-PAGE (as described in the Methods section) on CSFV E2 protein samples, gels were removed from the cassettes and the stacking gels detached and discarded. Separating gels were rinsed with dH_2O and placed in 10 mL of Coomassie Blue stain solution (recipe A2.25) for 30 minutes on a Belly Dancer Shaker. Gels were placed in 10 mL fresh destaining solution and shaken for an additional 3 hours before being washed with 20 mL MQH_2O. Gels were imaged on the BioRad Gel Documentation System and dried overnight between cellophane membranes.

Protein Quantification. CSFV E2 protein samples were quantified on Qubit (Invitrogen, USA) according to the Manufacturer's Qubit 3.0 Fluorometer Protein Assay protocol as described in the Methods section.

A1.2 Control Antigen Reactivity Assays

Control Antigen Arrays. Nitrocellulose membranes (BioRad, USA) were cut into strips for each of the sera and square grids added with pencil. Onto each array, 3 μL of *Brucella* LPS were spotted as the positive control and 3 μL of CSFV E2 protein for the negative control. All spots were allowed to dry completely for 10 – 15 minutes. CSFV E2 protein sample #2 and *Brucella* LPS samples were sourced from archived, in-house isolations suspended in phosphate buffered saline (PBS) and 50% glycerol.

Western Blotting. Western blotting assays on spotted nitrocellulose strips were carried out using the iBind Automated Western System (Life Technologies, USA) as described in the Methods section.

Chemiluminescence. BioRad's Clarity Enhanced Chemiluminescence (ECL) kit was used on spotted nitrocellulose strips according to manufacturer's specifications as described in the Methods section. ECL results were imaged on the BioRad Gel Documentation System using the chemiluminescence filter.

A1.3 Serological Reagent Validation Results

A1.3.1 CSFV E2 Protein Confirmatory Assays

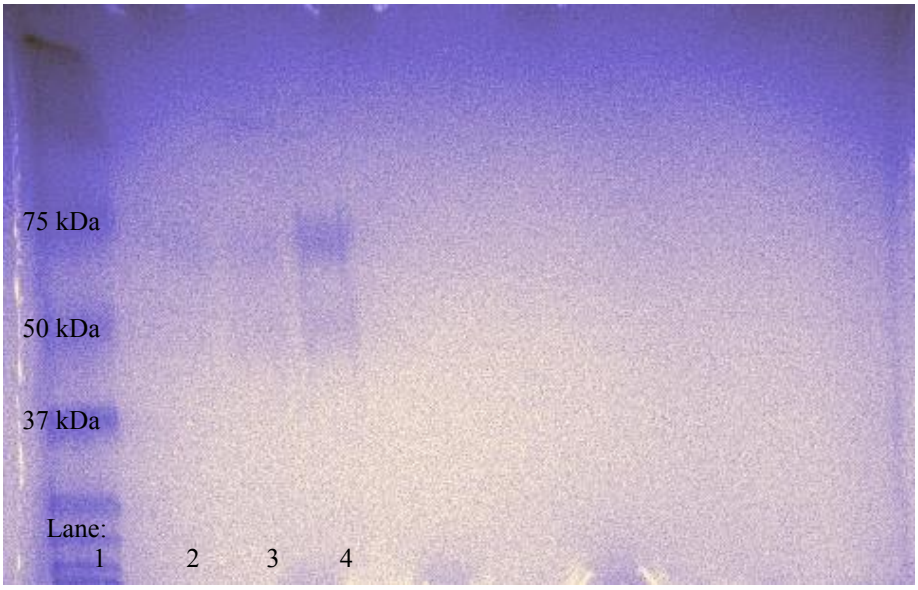


Figure 20: Coomassie Blue E2 Protein Stain Gel Image. Presence of CSFV E2 Proteins in in-house samples displayed. Image captured on BioRad's Gel Documentation System. Lane 1: Prestained 10-250 kDa Protein Standards (BioRad, USA); Lane 2: CSFV Protein Sample #2; Lane 3: CSFV E2 Protein Sample #3; Lane 4: CSFV E2 Protein Sample #4.

Table 9: E2 Protein Quantification Results. Quantification performed on Invitrogen's Qubit. Concentrations of CSFV E2 protein samples displayed. Proteins suspended in phosphate buffered saline (PBS) and 50% glycerol. Samples diluted in Qubit Working Solution (WS) for fluorometric analysis.

CSFV E2 Protein Sample	Concentration Following Purification (ng/µL)
#2	142
#3	221
#4	292

A1.3.2 Control Antigen Immunoreactivity Arrays

Table 10: Reagent Viability Assessment Results. Chemiluminescence detection outcomes from immunoreactivity assays between serum panels and antigen controls displayed demonstrating mutual functionality.Green coloured boxes represent condition success. Control antigens were exposed to each brucellosis positive and negative bovine serum. Sera A – D are brucellosis positive. Sera E – H are brucellosis negative. "+" symbols indicate reactivity detected by chemiluminescence. "–" symbols indicate no reactivity detected by chemiluminescence.

		Positive Control	**Negative Control**
	Sample	*Brucella* LPS Reactivity	CSFV E2 Protein Reactivity
Positive Sera	Serum A	+	–
	Serum B	+	–
	Serum C	+	–
	Serum D	+	–
Negative Sera	Serum E	–	–
	Serum F	–	–
	Serum G	–	–
	Serum H	–	–

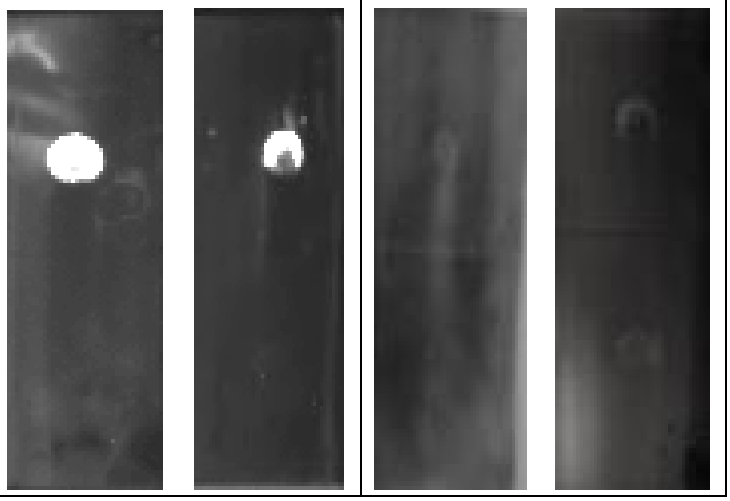
		Positive Sera		Negative Sera	
	Sample	Serum A	Serum B	Serum E	Serum F
Positive Control	*Brucella* LPS				
Negative Control	CSFV E2 Protein				

Figure 21: Reagent Assessment Membrane Image Data Samples. Preliminary reactivity results between sera and antigen controls displayed. Nitrocellulose membrane strip images captured on BioRad's Gel Documentation System with chemiluminescence filter following western blotting. CSFV E2 protein and *Brucella* LPS samples were sourced from archived, in-house isolations suspended in phosphate buffered saline (PBS) and 50% glycerol. Sera A and B are brucellosis positive; Sera C and D are brucellosis negative.

A1.4 Protein Purification Coomassie Blue Gel

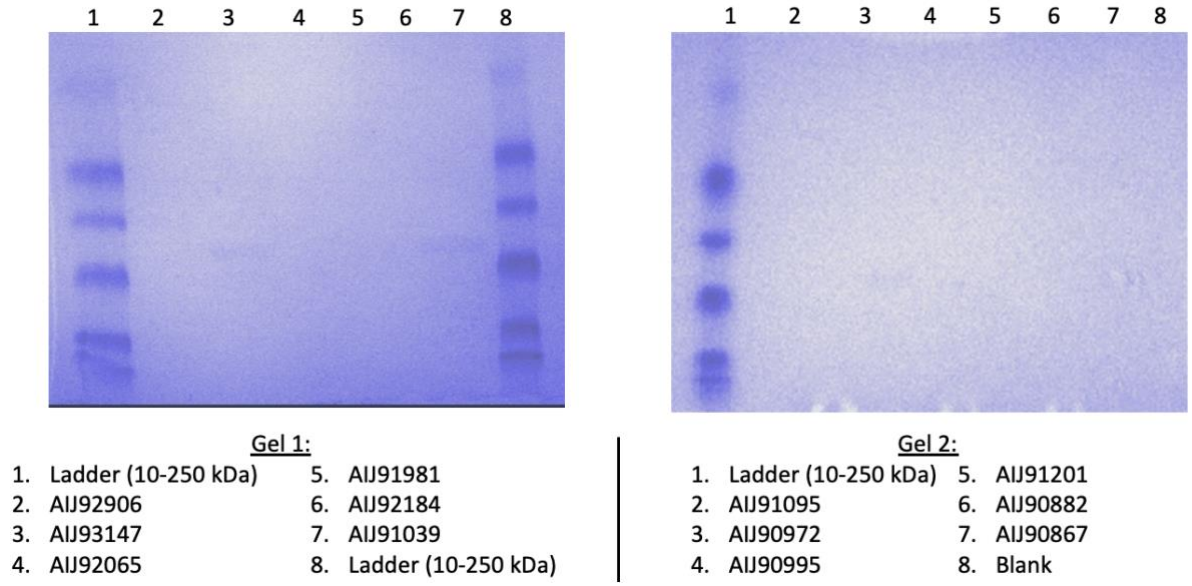


Figure 22: Coomassie Blue Gel Following Protein Candidate Purification. Polyacrylamide gels containing all twelve expressed and purified protein candidates dyed with Coomassie Blue stain following SDS-PAGE displayed.

A2: Reagent Recipes

A2.1: Tris-HCl (1 M, pH 8.0)

- 12.1 g Trizma base (Sigma, USA); sMQH$_2$O, brought to 100 mL; 12N HCl (Sigma, USA) by dropwise addition to adjust to pH 8.0

A2.2: dNTPs (10 mM)

- 10 μL of 100 mM dATP (Thermo Scientific, USA); 100 mM dCTP (Thermo Scientific, USA); 100 mM dGTP (Thermo Scientific, USA); 100 mM dTTP (Thermo Scientific, USA); 60 μL sMQH2O

A2.3: Agarose Gel Solution (1% w/v)

- 0.25 g agarose (Sigma, USA); 25 mL 0.5x TBE Buffer (54 g Trizma base (Sigma, USA); 27.5 g boric acid (Sigma, USA); 20 mL of 0.5 M EDTA (pH 8.0) (Sigma, USA); 1 L MQH$_2$O)

A2.4: Loading Buffer (6X)

- 0.025 g bromophenol blue (Sigma, USA); xylene cyanol FF (Sigma, USA); Ficoll 400 (Sigma, USA); sterile 0.5 M EDTA (pH 8.0) (Sigma, USA); 10 mL sMQH$_2$O

A2.5: Sterile LB Broth

- 25 g Difco^{TM} LB, Miller broth powder (Becton Dickinson (BD), USA); 1 L MQH$_2$O

A2.6: Sterile LB Agar

- 40 g Difco^{TM} LB, Miller agar powder (BD, USA), 1 L MQH$_2$O

A2.7: SOC Media Solution

- 2 g Tryptone (Sigma, USA); 0.5 g Yeast Extract (Sigma, USA); 0.05 g NaCl (Sigma, USA); sMQH$_2$O up to 100 mL

A2.8: Glucose (1 M)

- 1.8 g D-(+)-Glucose (Sigma, USA); sMQH_2O up to 100 mL; filter sterilized by passing the solution through a 0.22 μm filter (Millipore, USA)

A2.9: Carbenicillin (100 μg/ml)

- 1 g Carbenicillin Disodium Salt (Sigma, USA); 10 mL sMQH_2O; filter sterilized using 0.22 μm filter (Millipore, USA)

A2.10: $MgCl_2$ (80mM) – $CaCl_2$ (20 mM) Solution

- 1.6264 g Magnesium Chloride Dihydrate (Sigma, USA); 0.2940 g Calcium Chloride Hexahydrate (Sigma, USA); 1 L MQH_2O; filter sterilized by passing the solution through a 0.22 μm filter (Millipore, USA)

A2.11: $CaCl_2$ (0.1 M) in 10% Glycerol

- 9 mL 0.1 M CaCl2 (1.47 g Calcium Chloride Dihydrate (Sigma, USA); 1L MQH2O; filter sterilized by passing the solution through a 0.22 μm filter (Millipore, USA)); 1 mL Glycerol (Sigma, USA) sterilized by autoclave)

A2.12: Agarose Gel (1% w/v) Solution

- 0.25 g agarose (Sigma, USA); 25 mL 0.5x TBE Buffer (54 g Trizma base (Sigma, USA); 27.5 g boric acid (Sigma, USA); 20 mL of 0.5 M EDTA (pH 8.0) (Sigma, USA); 1 L MQH_2O)

A2.13: SDS-PAGE Sample Buffer #2 (2x)

- For 10 mL: 2 mL of 0.5 M Tris-HCl (pH 6.8) (30.275 g Trizma base (Sigma, USA); MQH_2O up to 500 mL; adjusted to pH 6.8 with 6 M HCl (Sigma, USA)); 4 mL of 100% glycerol (Sigma, USA); 2 mL of β-Mercaptoethanol (Sigma, USA); 0.4 g of SDS (lauryl sulfate) (Sigma, USA); 400 μL of 0.5% bromophenol blue (Sigma, USA); 1.6 mL of MQH_2O

A2.14: Separating Gel Solutions

- 4.0 mL 30% Acrylamide/Bis Solution (2.7% crosslinker) (BioRad, USA); 2.5 mL 1.5 M Tris-HCl – pH 8.8 (90.825 g Trizma base (Sigma, USA); MQH_2O up to 500 mL; adjusted to pH 6.8 with 6 M HCl (Sigma, USA)); 3.35 mL MQH_2O; 100 µL 10% SDS (10 g of SDS (lauryl sulfate) (Sigma, USA); MQH_2O up to 100 mL); 50 µL 10% APS (1 g Ammonium Persulfate (BioRad, USA); MQH_2O up to 10 mL); 5 µL TEMED (Sigma, USA)

A2.15: Stacking Gel Solution

- 0.65 mL 30% Acrylamide/Bis Solution (2.7% crosslinker) (BioRad, USA); 1.25 mL 0.5 M Tris-HCl – pH 6.8; 3.05 mL MQH_2O; 50 µL 10% SDS; 25 µL 10% APS; 5 µL TEMED (Sigma, USA)

A2.16: SDS-PAGE Running Buffer (1x)

- 100 mL of 10x SDS-PAGE Running Buffer (Sigma, USA); 900 mL of MQH_2O

A2.17: Protein transfer buffer

- 5.82 g Trizma base (Sigma, USA); 2.93 g Glycine (Sigma, USA); MQH_2O up to 1L

A2.18: iBind Solution (1x)

- 6 mL of iBind 5x Buffer (Life Technologies, USA); 300 µL of 100x Additive (Life Technologies, USA); 23.7 mL MQH_2O

A2.19: Diluted 1° Antibody (Penta-His)

- 10 µL Penta-His (Invitrogen, USA); 2 mL of 1x iBind Solution (Life Technologies, USA)

A2.20: Diluted 2° Antibody (HRP Anti-Mouse IgG)

- 10 µL Horseradish Peroxidase (HRP) Conjugated Goat Anti-Mouse IgG (Jackson ImmunoResearch, USA); 2 mL of 1x iBind Solution

A2.21: Wash/Equilibrium Buffer (WEB) (pH 8.0)

- 6.9 g of 50 mM $NaH_2PO_4.H_2O$ (Sodium phosphate monobasic monohydrate) (Sigma, USA); 17.4 g of 300 mM NaCl (Sigma, USA); 1.36 g of 20 mM imidazole (Sigma, USA); Stirred well and adjusted to pH 8.0 with 10 N NaOH (Sigma, USA), brought to 1 L with MQH_2O),

A2.22: Elution Buffer B (pH 8.0)

- 6.9 g of 50 mM $NaH_2PO_4.H_2O$ (Sodium phosphate monobasic monohydrate) (Sigma, USA); 1.36 g of 250 mM imidazole (Sigma, USA); Stirred well and adjusted to pH 8.0 using 10 N NaOH (Sigma, USA), brought to 1 L with MQH_2O)

A2.23: Diluted 1° Antibody (Bovine Serum Samples)

- 200 μL of in-house Brucellosis positive or negative serum preparations; 2 mL of 1x iBind Solution (Life Technologies, USA)

A2.24: Diluted 2° Antibody (HRP Anti-Bovine IgG)

- 10 μL of HRP Conjugated Goat Anti-Bovine IgG (Jackson ImmunoResearch, USA); 2 mL of 1x iBind Solution

A2.25: Coomassie Blue Stain Solution

- 2.5 g Coomassie brilliant blue (Sigma, USA); 200 mL Methanol (Sigma, USA); 50 mL Acetic Acid (Sigma, USA); 250 mL MQH_2O) for 1 hour on a Belly Dancer Shaker (IBI Scientific, USA). Gels were removed and placed in 10 mL of destaining solution (400 mL Methanol (Sigma, USA); 100 mL Acetic Acid; 500 mL MQH_2O

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