# **Application of a Whole Genome Approach to the High Throughput Discovery of Novel Diagnostic Antigens for *Brucella abortus***

**Teresa Nguyen**

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Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

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## Abstract

*Brucella abortus* is the etiological agent of bovine brucellosis, a zoonotic disease that can be transmitted to humans through direct contact with infected animals or consumption of contaminated food products. Current serological tests used to identify infected animals rely on the detection of antibodies to O-antigens of the smooth lipopolysaccharide (sLPS) of *B. abortus*. Due to the presence of structurally similar O-antigens of bacteria such as *E. coli* O:157 and *Yersinia enterocolitica* O:9, these tests can produce false-positive results, which requiring alternative protein target antigens. We hypothesize that through comparative genomics and bioinformatics analysis of all ORFs within the *B. abortus* genome, followed by profiling of the humoral immune responses to surface and extracellular proteins, novel protein antigens with diagnostic potential may be discovered. In this study, the genomes of thirteen strains were analyzed using a subcellular localization prediction database (PSORTb) to identify proteins in the outer membrane and extracellular space. A total of 100 ORFs coding for such proteins including known immunogenic proteins reported in literature were identified and selected for recombinant protein expression using high-throughput *in vivo* cloning and *in vitro* transcription/translation strategies. The *in vitro* expression of 67 of these candidates has been successfully demonstrated. These recombinant *B. abortus* proteins were subsequently probed with *E. coli* pre-adsorbed sera from infected animals for the identification of immunoreactive protein antigens. Ten unique candidates were demonstrated to be antigenic and have the potential for diagnostic applications. This study illustrates a unique, high throughput strategy to express and screen proteins of a bacterial pathogen for novel diagnostic antigen discovery.

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## List of Abbreviations

**A**

**APHIS:** Animal and Plant Health Inspection Service

**B**

**BSS:** Bovine Surveillance System
**BSA:** Bovine Serum Albumin

**C**

**cELISA:** Competitive Enzyme-Linked Immunosorbent Assay
**CFIA:** Canadian Food Inspection Agency
**ChrI:** Chromosome I
**ChrII:** Chromosome II

**D**

**DNA:** Deoxyribonucleic Acid
**dsDNA:** Double-stranded DNA

**E**

**EB:** Elution Buffer
**ELISA:** Enzyme-Linked Immunosorbent Assay
**ER:** Endoplasmic Reticulum

**F**

**FAO:** Food and Agricultural Organization
**FPA:** Fluorescence Polarization Assay
**FPSR:** False Positive Serological Results

**G**

**GFPuv:** Green Fluorescent Protein variants

**H**

**HRP:** Horseradish Peroxidase

**I**

**IgG:** Immunoglobulin G

**L**

**LB:** Luria-Bertani
**LIC:** Ligation-Independent Cloning
**LPS:** Lipopolysaccharides

**M**

**mAb:** Monoclonal Antibody
**Mbps:** Mega Base Pairs

**N**

**NCBI:** National Center for Biotechnology Information
**NEB:** New England Biolabs
**NGS:** Next-generation Sequencing

**NT:** Nucleotide

**O**
**OIE:** World Organization for Animal Health
**OLF:** Ottawa Laboratory Fallowfield
**OM:** Outer Membrane
**OMPs:** Outer Membrane Proteins
**ORFs:** Open Reading Frames

**P**
**PBS:** Phosphate-Buffered Saline
**PBST:** Phosphate-Buffered Saline, Tween
**PBS-TT:** Phosphate-Buffered Saline, Tween, Triton
**PCR:** Polymerase Chain Reaction

**S**
**SDS-PAGE:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
**S-LPS:** Smooth-lipopolysaccharides

**U**
**USDA:** United States Department of Agriculture

**W**
**WHO:** World Health Organization

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# 1 INTRODUCTION

*Brucella abortus* is a gram-negative, endospore-forming coccobacilli. An intracellular facultative anaerobe, it is the etiological agent of bovine brucellosis. Bovine brucellosis can cause significant economic losses due to the increased spontaneous abortions in cattle and the decrease in exports and sales.

While primarily affecting bovine populations, *Brucella abortus* is also a zoonotic pathogen. Humans can acquire the bacterial infection through direct contact with diseased animals as well as ingestion of contaminated meats. The pathogen can enter the body through mucous membranes, and abrasions of the skin. The major threat to the public health comes from contact or consumption of food products contaminated with the pathogen, largely unpasteurized products such as milk and cheese. Particularly alarming has been its potential for human-to-human transmission, with congenital cases recorded(1, 2). *B. abortus* has a low fatality rate but its danger lies in its initial subclinical conditions that may evolve into a chronic disease (3), with severe symptoms such as endocarditis and arthritis.

*Brucella* is identified conventionally by culture plating, isolation and subsequent biochemical tests. This type of testing requires very specific techniques, tedious procedures, and level 3 biocontainments. Delivery of test results may then be delayed should samples need to be sent to distant labs. Successful isolation of the pathogen also depends on the stage of disease due to different levels of viable bacteria present, affecting detection rates (4, 5). Broth culturing for enrichment must be performed in order to improve the sensitivity of detection, but the time needed for results to be delivered is problematic for both the farming industry and food safety.

Serological tests have been proven to be a simple and inexpensive alternative with more rapid results(6), leading to their recommendation for large-scale surveillance despite the qualitative nature of such tests as the agglutination test. Serological tests for detecting antibodies to specific smooth-lipopolysaccharides (S-LPS) are widely used for the presumptive identification of infected animals.

The S-LPS may play an important role in the bacterium's pathogenesis, although its precise role is not completely defined. It may be potentially involved in invasion and intracellular multiplication as well as countering complement-mediated lysis(7). The O-chain is one of the most significant portions of the S-LPS. These chains themselves are largely involved with blocking complement-mediated killing of the bacteria by inhibiting complement deposition(8). The importance of S-LPS is highlighted by its role as a major antigen(7) targeted for in the design and development of most serological tests used currently.

Amongst the more commonly used serological tests are serum agglutination, Rose Bengal, and complement fixation tests. These tests cannot be considered adequate stand-alone assessments because of their low specificity. Other tests such as the fluorescence polarization assay have good specificity, but

poor sensitivity and are thus recommended as screening or confirmatory tests. However, they can be used as a standalone test if no substitute is available (9). Similar O-antigens are found in other bacteria such as *Y. entercolitica* O:9 and *E. coli* O157(10), leading to cross-reactivity and false positive serological results (FPSR). With the increasing rate of infection in shared reservoirs, exhibited by *Y. enterocolitica*(11) raising the rate of FPSR (12), a pressing need for tests with greater specificity has been demonstrated. Similarly, the *B. abortus* S19-vaccinated cattle elicit a humoral immune response to the same O-antigen that the pathogenic strains have, further complicating the serological identification of diseased cattle (13).

As more research continues to point to the O-chain as the major determinant of a strong protective antibody response, different serological tests must be developed in order to discriminate between vaccinated and diseased cattle. Protein antigens are being considered as an alternative to S-LPS. Past research in this area has been limited due to the sheer number of proteins available for testing. A potential solution is the use of bioinformatics tools to predict localization of the proteins encoded by the genome of *B. abortus*, thus prioritizing those more likely to be immunogenic. Outer membrane and secreted proteins have been identified as having a high potential for eliciting antibody responses during infection because of their exposure to the immune system of infected animals. For the purposes of this study, the proteins predicted to be located in the extracellular space are hypothesized to be have been secreted(14) and may be immunogenic.

In the 1980s, a subset of outer membrane proteins (OMPs) being considered major outer membrane proteins were identified and separated into groups by their size(15). Many of these proteins were dismissed as weakly or non-immunogenic(16). However, as whole genome sequencing has resulted in complete genome sequences of many strains of *B. abortus* being available, our ability to assign localizations to proteins has expanded to include hypothetical proteins through bioinformatics(17). This expansion could lead to the identification of new potential immunogenic proteins beyond the original scope explored decades earlier.

## 1.1 Rationale of Study

None of the current tests are ideal and cannot be considered adequate stand-alone assessments; they must be used with others for the delivery of a confident diagnostic result. These tests also have low specificity due to the target LPS O-chains with the similarity to that of *Yersinia entercolitica* O:9 and *E. coli* O157, leading to cross-reactivity and false positive results. Similar symptoms such as weakened calves may also incorrectly confirm a brucellosis diagnosis, although *Y. entercolitica* and *E. coli* also often present gastrointestinal symptoms.

Protein antigens are being considered as an alternative to S-LPS. Past research in this area has been limited due to the sheer number of known proteins available for assessment and the difficulty identifying and isolating ideal candidates. For those that have been investigated to date, different studies

have resulted in conflicting views towards their usability in diagnostics. A potential solution to this problem has been the use of bioinformatics tools to predict localization of the proteins, thus prioritizing those more likely to be immunogenic. Outer membrane and secreted proteins have been identified as having a high potential for antigenicity because of their surface exposure or extracellular nature (18), being similarly located comparing to the O-antigens (Figure 1). This idea could potentially lead to the identification of novel antigens for superior specificity and sensitivity(19). By using bioinformatics tools, identifying these surface-exposed proteins can result in a pool of candidates for the assessment of their antigenicity by experimental approach.

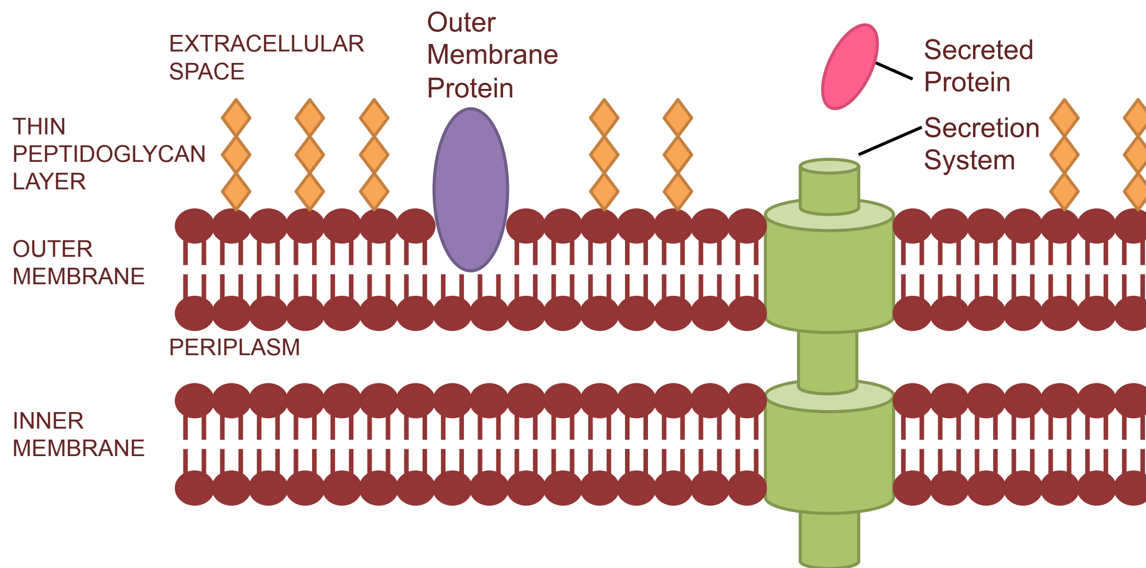



*Figure 1. A schematic representation of the cell envelope of B. abortus with location and surface exposure of proteins.*

## 1.2 Hypothesis

Through comparative genomics and bioinformatics analysis of all open reading frames (ORFs) within the *B. abortus* genome, followed by profiling the humoral immune responses to the surface and extracellular proteins identified by bioinformatics, novel protein antigens with diagnostic potential may be discovered.

## 1.3 Objectives

1. To identify all ORFs coding for extracellular and outer membrane proteins using bioinformatics tools.
2. To assess the reactivity of the recombinant proteins encoded by identified ORFs with sera from *B. abortus*-infected animals using a high throughput approach (*in vivo* cloning, *in vitro* transcription/translation, and protein arrays).

# 2 LITERATURE REVIEW

## 2.1 Brucellosis Overview and Impact

The beef industry plays a prominent role in the Canadian economy, contributing $33 billion worth of sales of goods and services(20). As a valuable commodity, the Canadian government has taken great strides in order to protect this food supply, creating a Canadian Beef Cattle On-Farm Biosecurity Standard. Under this standard, there are diseases of concern listed that may have a devastating impact on the industry as well as on animal or human health if they are not carefully monitored, managed, and controlled. One such disease is brucellosis.

Bovine brucellosis, caused by *B. abortus*, is a disease associated with massive consequences that has prompted the development and enforcement of strict regulations for the relocation of cattle, both internationally and nationally(21). The clinical symptoms of bovine brucellosis include infertility and spontaneous abortion. Infected cows are three to four times more likely to suffer from an abortion than healthy cows(22). As females remain carriers, the bacterium is capable of propagating and transmitting to future offspring while also remaining viable in milk. While predominantly in female cattle, males are not immune from symptoms and repercussions. They may develop testicular infections, leading to a further reduction in fertility. A farm's overall profitability is then impacted by a decrease in exports and sales of food products followed by costly replacements of the affected members in the herd and the lost progeny.

Furthermore, *B. abortus* is labelled as a bioterrorism agent. In addition to the potential for great economic losses and threat to the food supply, its direct health impact on humans cannot be overlooked. While brucellosis has been reported as early as the early 1850s, there remains a lack of vaccines available for the human population(23). Paired with the difficulty in correctly identifying the organism, *Brucella* has the potential for devastation with inappropriate use. In fact, this has been highlighted by past research into and subsequent weaponization of the bacteria by the United States of America in the 1950s, a first at the time for the country(23). Following the weaponization of *B. suis*, programmes were initiated to further study the potential with other species (24). Due to the low infective dose of only 10-100 cells(23) and its stability and transmissibility through both aerosols and food contamination(25), the economic impact two decades ago was suggested to be over $450 million per 100 000 exposed(26). These costs include the lost productivity from afflicted people missing work(27), weakening the economy as a whole. Because of the ease with which *Brucella spp*. can spread to cause serious problems in economy and public health, research must be done in order to minimize bacterial dispersion and subsequent impact. Such research could include the identification of novel antigens for the development of diagnostic tests and vaccines.

## 2.2 Bacterial Classification and Genome

### 2.2.1 Taxonomy

*Brucella abortus* is a gram-negative, endospore-forming coccobacilli usually arranged as a singlet. It is small (0.5-0.7 $\mu$m × 0.6-1.5 $\mu$m) facultative intracellular pathogen capable of surviving within the host(24), which contributes to its virulence. While *B. abortus* is primarily located in cattle, there are also other species of *Brucella* that are highly similar genetically(28). Yet each species has its own primary host and despite similar genomes, different immune responses are evoked and different proteins are expressed(29). The expression of varying outer membrane proteins has been associated with the differences in host tropism, coming from short open reading frames (ORFs) as well as large insertions-deletions. Four of these species (*Brucella abortus*, *Brucella melitensis, Brucella suis,* and *Brucella canis*) have a known ability to cause disease in humans(24).

The nucleotide sequence similarities and differences coincide with the phylogenetic relationship of *Brucella* species based on restriction fragment length polymorphism, beneficial when genomes weren't completely sequenced. This counters the theory that all the species are simply differing biovars of *B. melitensis*(30). The time of divergence between all the classic species does appear to be near-simultaneous, as indicated by short internal branches(31). A gene conversion in the *omp2* locus led to a separation between *B. melitensis* and *B. abortus* from the other species(30), resulting in shared fragments between the two(32).

Homoplasy may also explain the similarities seen between the species, which are known to be independent mutations rather than inherited traits(33). Identical profiles were seen when some comparison between isolates that were separated both geographically and temporally were made(34), which is best explained by identical evolution.

*Brucella abortus* itself has eight biovars: 1 to 7, and 9 (35). They are separated based on serological properties as well as responses in culture(36), including CO2 requirements, H2S production, and dye sensitivity(37). The most reported cases are from 1, 2, 3, 4, and 9(22), with biovar 1 being the most significant, accounting for more than 80% of isolates identified worldwide(38).

### 2.2.2 Genome Composition and Organization

The bacterium has two circular chromosomes of dramatically different sizes, with chromosome I (chrI) 2.1 Mbp and chromosome II (chrII) 1.2 Mbp (30). Both chromosomes have a G/C content of approximately 57%(32). The differences between the two are important in that they seem to describe an evolutionary event that offered an advantage over time. Chromosome I has an origin of replication similar to what is usually seen in bacterial chromosomes, while that of chromosome II appears more plasmid-

like(32). Likely associated with its larger size as well as its more characteristic bacterial nature, chromosome I contains most of *B. abortus'* essential genes.

In the past, genome analysis using techniques such as polymerase chain reaction (PCR) has been used to identify and subsequently group species and biovars by detecting genomic changes. In particular, *B. abortus* is differentiated from the other *Brucella* species by an inversion in chrII(32) but other indels have been noted. Strains of biovars such as *B. abortus* biovar 1 have been differentiated through multilocus variable nucleotide tandem repeat analysis, which allowed for genotyping of the species(32).

The *B. abortus* genome is considered large relative to other bacterial pathogens, which offers an explanation for the bacteria's ability to adapt to other environments and different hosts(39). The two chromosomes contain over 3000 ORFs(32), although chrI is where the majority of essential genes are located(32).

Notably, *B. abortus* cells do not have the classical virulence factors ordinarily seen in pathogenic species, such as flagella and type III secretion systems(40). In fact, they lack factors that are known to harm eukaryotic cells(41). For instance, exotoxins or any inducers of apoptosis are absent in *Brucella*. Instead, *B. abortus*' virulence appears to stem from the bacterial ability to adapt to a wide variety of environments, particularly through adaptation of the cell envelope. It is then able to avoid destruction, allowing it to proliferate throughout the body.

### 2.2.3 Cell Structure in Virulence

The structure of *B. abortus* cells is a key component of its virulence. The cell envelope consists of the outer membrane, the periplasmic space, and the inner membrane. It has been identified over the years as a complex structure, which is in part a result of its large genome with over 3000 ORFs. This nature gives the bacterium options in terms of varying protein expression, which are employed when remodelling their cell envelopes to survive intracellularly. For example, upon entering the macrophage, the bacterium induces the expression of 42 proteins and decreases that of over 100 others(42). Not only is remodelling possible, these changes are reversible and are essential to the interactions that *B. abortus* has with host proteins and processes(40).

Different stages in the infection cycles affect the microenvironments that the bacterium will encounter. This can be attributed to the fact that cell envelopes are able to adapt using the expansive library of ORFs, changing proteomes as the infection cycle progresses for their continued survival(40).

## 2.3 Clinical Disease

### 2.3.1 Infection Progression

The proteomic changes in the brucellae's cell envelope appear to correspond with the stages of the infection cycle(43). As different stages of infection result in changes to body conditions, the protein

expression profiles change to reflect the requirements of the new environment(44). For example, a change in oxygen levels lead to an adjustment in respiration pathways used(44).

Upon entry into the host's system, the brucellae are engulfed through phagocytosis and reside inside the early phagosomes(23). While the mechanism isn't completely understood, *B. abortus* is capable of disrupting the fusion between phagosome and lysosomes thus preventing its own destruction and can then reproduce within the phagosome(24).

By taking control of intracellular trafficking, the bacterium is capable of directing itself to bind with the endoplasmic reticulum (ER) cisternae. The ER supplies some of the required nutrients needed for bacterial growth(22). This allows the bacterium to remain in a low stress environment, without the use of major stress response molecules such as bacterial oxidative response proteins and chaperones(44). Eventually, low oxygen levels initiate a switch to anaplerotic routes and catabolic pathways. These pathways and routes allow the bacterium to generate molecules essential to survival. The host cell must remain alive, which involves cellular mechanisms such as the VirB type IV secretion system and an apoptosis inhibitory mechanism(44).

Being engulfed within the early phagosome also offers a setting conducive to replication, which continues for up to 48 hours. After 20 hours of infection, the environment has again been altered in order to support protein synthesis. Following the infection, activity of amino acid, peptide, and iron transporters has been boosted to increase amino acid catabolism. Meanwhile, the Omp2b porin, known to be involved with outward growth, declines. Inversely, the Omp2a porin, which is nearly homologous to the Omp2b porin but forms a larger pore, is better able to contribute to intracellular growth as its size is advantageous when competing for nutrients(45). Echoing this change, the topology of the outer membrane has been modified to make use of genes involved in biosynthesis and metabolism(46). Despite the increased activity within the macrophage, the cell does not show any indication of infection.

After 44 hours, profiles tend to revert to their original state as maximum replication within the macrophage has been achieved and replication thus plateaus.

Armed with this knowledge on disease progression, the time course between 3 hours and 20 hours post-infection for bacterial research are often selected to optimize recovery of intracellular bacteria to study critical bacterial adaptation. The proteomic changes involved in *Brucella* replication and persistence are reflected in the clinical presentation of disease and the recovery of bacteria may also lead to the identification of proteins on the outer membrane that can be used as diagnostic antigens. After establishment of the bacterium within a macrophage, they are able to lie dormant and occasionally released to cause chronic disease.

### 2.3.2 Disease in Cattle

An abnormally high number of abortions in a herd often triggers a *B. abortus* investigation, making it one of the brucellosis' most recognizable clinical manifestations. This can be associated with the preferential establishment of the infection in the reproductive tract(24). Also increased are premature birth rates which lead to lowered milk production. Calves that make it to gestation are not immune to the effects of brucellosis. Newborns of infected cows are generally weaker and more susceptible to neonatal death(47).

While the most obvious effects on the growth and fertility of the herd are seen in the females due to the brucellae's propensity for affecting their reproductive nature, fertility in males can also be affected. Orchitis and epididymitis may lead to reduced semen quality, which can contribute to lowered fertility in the herd. Consequently, should this become a chronic symptom, the bulls can develop permanent infertility(47).

Decreased milk production, weak offspring, weight loss, and infertility are symptoms associated with the infected herd, making brucellosis one of the most serious livestock diseases(23). While sick animals are quarantined from the herd, heifers often serologically-negative until just after giving birth and subsequently spread bacteria.

### 2.3.3 Disease in Humans

The infection presents itself within two weeks in about 75% of patients(48) but brucellosis must continue to be monitored long after as a latency period of several months has been seen in some of the remaining patients. The bacterium manifests as an acute disease with generic symptoms such as general malaise, exhaustion, weight loss, and headaches seen in over 90% of cases(49), appearing much like a flu. Being synonymously known to have an undulant fever, patients frequently complain of a cyclic pattern of fever recovery and relapse over the span of several days; patients often feel improved in the morning before symptoms re-emerge throughout the day(24).

When fever is not present, brucellosis is difficult to diagnose through other symptoms alone and is often overlooked in non-endemic regions. Bone and joint pain throughout the body are fairly common, with the rare event of joint swelling. Without treatment, recovery may take weeks or months(24). Major symptoms vary depending on the location of the bacteria, leading to potential issues with the musculoskeletal, cardiovascular, and central nervous systems.

Acute disease varies greatly from one patient to another. Brucellosis can last from several days to over one year. This acute illness is capable of progressing to be chronically incapacitating with further complications, which occurs in 20 to 60% of infected people(24). Once *Brucella* enters the body, it is capable of spreading systemically via the blood and lymphatic system and creating a localized infection in any area of the body. In this manner, the bacterium in the blood is then able to spread to other organs and

subsequent symptoms will differ depending on the affected area. The gastrointestinal region is significant in terms of a target for the pathogen, affecting approximately 50% of patients. Less common are neuropsychiatric symptoms that can arise from infection of the nervous system, with side effects being as mild as headaches to as dangerous as seizures.

With symptoms ranging from neuropsychiatric to gastrointestinal to neurological, the severity of the disease is far-reaching and all-encompassing. Complications, especially those related to heart conditions, can lead to death. The true danger of brucellosis is because of its chronic phase, where eradication is particularly difficult. Humoral immunity and most antibiotics are incapable of eliminating the bacterium as it is intracellular(33). Due to the grievous effects of the transmissible disease, the zoonotic *B. abortus* cannot be overlooked even if its preferred host is not humans, as it is one of the most common zoonotic infections in the world.

## 2.4 Epidemiology

### 2.4.1 Hosts

While rare in countries such as Canada, the United States, and Britain, the disease remains endemic in many other regions including Latin America and the Mediterranean with prevalence approaching 68.8% in some herds in Africa(50). In these areas, the brucellae can be found in both domestic and wild animals, although the *Brucella* species do tend to have specific host preferences. Four species are zoonotic, although humans are not their primary reservoirs: *B. melitensis* primarily infects sheep and goats, *B. suis* targets swine, *B. canis* causes disease in canines, and lastly *B. abortus* is found in cattle.

*B. abortus* itself appears to favour infection in bovine species, particularly in cattle. As mentioned earlier, abortions are the main indicator of disease, but also aid in the spread of the bacterium, as they prefer to multiply in the placental and foetal fluids. These same fluids are excreted during abortions and can contaminate stables and farmyards, exposing the rest of the herd to the pathogen. Even a regular birth from an infected cow is capable of prompting an outbreak, as the bacterium, shed during delivery, can be transmitted to other cattle. The persisting presence of brucellae in the cow's uterus can lead to a spontaneous re-mergence if infected cattle are overlooked and left in the herd(51).

### 2.4.2 Prevalence & Risk Factors

Prevalence of bovine brucellosis varies throughout the world, with rates varying from 0.85% to 23.3%(23). It is difficult to determine values with certainty, as there are factors that can influence prevalence in a wildlife species that wouldn't ordinarily demonstrate such rates of incidence. For example, the presence of an asymptomatic reservoir can lead to a possibility of brucellae transmission, as

continued contact will naturally lead to more opportunities for infection even in a species that would generally not maintain the disease(52).

There are some clear influences on the progression and consequences of infection. Age is important although a range has not been clearly defined, with calves usually testing seronegative relative to other exposed sexually mature cattle, indicating decreased susceptibility to the bacterium(51). This seronegativity can be related to the bacteria sheltering in the lymph nodes and uterus up until a cow gives birth. Due to the major symptoms manifesting in the reproductive system, the disease is believed to target sexually mature animals, which helps explains the higher seroprevalence in older cattle(23).

Sex also plays a role in terms of severity of disease, although more in terms of their ability to spread the disease. It appears as though females have a higher infectivity rate, particularly when pregnant as the uterus is able to sustain the brucellae(51). This is further evidenced by links between earlier pregnancies and longer incubation periods, with abortions normally occurring after the 5$^{th}$ or 6$^{th}$ month of pregnancy(51).

Certain times of the year are associated with higher risks for epidemics, which differ between species. For example, peak times for disease spread in animals are often associated with their respective delivery cycles, while humans demonstrate higher rates of infectivity in the summer(23).

### 2.4.3 Intraspecies Transmission

The proximity of the cattle as well as their housing conditions creates an environment that favours transmission. For example, not only is the aborted fetus a source of brucellae but the subsequent uterine discharge can continue to contain the bacterial pathogen for up to fifteen days(51). This contamination of the cowshed or pasture is significant as brucellae can remain viable for long periods in varying conditions(22) and often results in brucellosis in other herd members as they move around their habitat. As a result, decontamination steps include heat killing at 56-61°C of fecal-infected bedding for 4.5 hours(51). The nature of cattle, between calving with other herd members around and licking aborted fetuses, only increases the likelihood of infection(53).

The udder is often permanently infected and may continue to shed brucellae sporadically in future milkings(24). The spread of the disease has been previously associated with the use of the same teat cups for milking between different cows(22).

Vertical transmission has been documented as within the infected pregnant population, leading to that 60-70% of subsequent fetuses carry the infection(22). This transmission can occur *in utero* or even after birth, through suckling from infected cattle(51).

Surveillance is hindered by this chronic nature of the disease(54). For example, a small percentage of infected calves remain infected well into adulthood while remaining serologically negative(22). In fact, they test seronegative usually until their first pregnancy(55). At this point, the cow

experiences an abortion or premature birth due to the infection and the propagation of brucellae during parturition once again exposes the herd.

Because of the ease with which *B. abortus* can spread through a herd, the trading and movement of cattle is strictly monitored in order to limit the potential introduction of brucellosis into disease-free herds and in fact, quarantined cattle are restricted from such measures(56). This is particularly important due to the nature of sales done at livestock fairs and shows.

Due to these regulations, risks now mainly arrive from unrestricted populations such as those found in the wild that may neighbour farms. In the past, co-mingling between domestic and wildlife herds has been documented, increasing direct contact as well as the potential for exposure to the aborted fetuses and afterbirth of an infected wildlife herd(34). Water sources have also been recognized as source of the bacterial pathogen, from direct contamination or rain run-off from contaminated areas(24). This same source of infection can pass the bacterium to other species as well.

### 2.4.4 Interspecies Transmission

Despite the favoured hosts of each species, interspecies transmission can occur when in contact with an infected host, resulting in infection(51). In fact, between the six different species of *Brucella*, nearly all animal species can develop brucellosis(23). Some studies have even shown that under the same conditions, the transmission rate of *B. abortus* between cattle and bison is statistically similar to that seen between only cattle(57), although the transmission appears to be self-limiting once in a secondary species.

Nevertheless, the zoonotic nature of *B. abortus* is alarming to human health and safety as humans can acquire the bacterium and become infected. There are numerous routes of infection available for the bacterium to infect humans, including through the digestive tract, lungs, mucosal layers, and the skin(23). While airborne transmission has been reported, *B. abortus* is primarily acquired through direct contact with the infected host, including its secretions.

As such, close proximity plays a major role in transmission which lends a higher risk of infection to certain professions. Farm occupational groups, including veterinarians and farmers, are more exposed due to their direct contact with infected animals as well as their continued presence in contaminated surroundings(24). Within these particular groups, the bacterium generally spreads through the mucous membranes of the respiratory and gastrointestinal tract, as well as abrasions of the skin. Also at risk are those who process animal products in abattoirs as well as laboratory staff who study brucellosis or work with live vaccines because of the potential for self-inoculation and aerosolization of cultures, which has been observed in laboratory accidents(58). In fact, *Brucella spp.* have been identified as the bacterium most commonly involved in laboratory incidents(59).

Outside of occupational risks, the general population may still become ill in large part due to ingestion of infected food products. Unpasteurized milk or other milk products from infected cows are a

common source of infection(51), as the bacterium may proliferate in the milk ducts. Unpasteurized products such as butter, cream, or ice cream may even be made with such a process where the brucellae in contaminated milk are concentrated and persist for several months(24). Risks with meat are more common in countries where such dishes may be eaten raw or undercooked(24).

Of importance are the cases of human-to-human transmission, as interspecies transmission must first occur due to the lack of a *Brucella spp*. with humans as its main host. The majority of these are from laboratory workers being infected by a patient's samples. There have been the rare occasions where babies become infected transplacentally or through their mother's breast milk(1). Otherwise, human-to-human transmissions are limited to blood or organ transfers(24).

While different *Brucella* species are zoonotic, humans are considered an epidemiological impasse, where brucellae can spread between the population but not to another host species.

## 2.5 Outbreak History

### 2.5.1 Eradication

Upon initiation of the eradication program in the 1940s, a vaccination schedule was put into place(60) in order to reduce the infected population to a manageable level that would allow for a practicable and cost-effective test-and-slaughter process. *B. abortus* strain 19, the live vaccination strain, was given to calves to stimulate antibodies that offer partial protection, although 60 to 70% of those vaccinated animals were completely protected. By protecting young calves, the brucellosis-infected adults could be removed without decimating the herd.

The success of the Canadian program was recognized worldwide when the country was declared brucellosis-free in 1985, with sporadic lone cases over the years(60). The last documented outbreak was in 1989(61), but was quickly contained.

### 2.5.2 S19 Live Vaccine

As a live attenuated vaccine, *B. abortus* S19 was isolated in 1923 from a Jersey cow and was originally virulent. However, after being accidentally left at room temperature for a year, the bacterium was determined to be of a lower virulence in the guinea pig test subjects. Further testing in cattle demonstrated that *B. abortus* S19 possessed low pathogenicity, high immunogenicity, and moderate antigenicity(62). These attributes allowed it to be used successfully for over half a century(42). The vaccine prompts the development of antibodies targeting the lipopolysaccharide (LPS) O-chain, an immunodominant antigen. In order to prevent the interference of these antibody titres with diagnostic tests, which detect the same antibodies to indicate the presence of infections, this live vaccine must be administered between three to eight months of age to allow for residual LPS antibody levels to decrease to titres that don't interfere with antibody tests, preventing false positives.

Another major problem with S19 is that, although infrequent, cattle may be chronically infected and demonstrate the same symptoms as if infected with a field strain. As a result, once substantial vaccination was achieved and the test-and-slaughter program was introduced, the government of Canada reduced and finally discouraged the vaccine strategy. However, this vaccine is still used in countries that may import their food products into Canada and as such, testing methods need to be improved in order to reduce the economic impact of a food recall based on a false positive.

### 2.5.3 Re-emergence

While eradicated in several countries, bovine brucellosis has historically re-emerged in such countries when surveillance was unable to adapt to changes in socioeconomic, political, and technical factors. For example, the islands of Fiji underwent a campaign of vaccinations combined with test-and-slaughter amongst domestic herds for brucellosis. The success of this campaign and subsequent declaration of eradication in 1996 was justified after having gone six years without a reported case. However, thirteen years later in June 2009, a surge in abortions in cows was observed and lead to a quarantine of a farm. In the end, more than half a dozen farms and over 9000 cattle were implicated in the outbreak.

While the exact source of the outbreak has still not yet been determined, Fiji's strict laws prohibiting the import of cattle suggests the bacterium was already present in the country. This leads to the possibility that the disease arose from a chronically infected member of the herd or from an untested herd or an unknown reservoir.

Prior to eradication, brucellosis outbreaks in Canada were fairly limited to wild herds. Bison of the Elk Island National Park grew from 37 positively identified infections to 111 as well as 34 suspicious cases from 343 tested animals from 1946 to 1947. The bacterium was also isolated from elk of the same park in the same study(52). Because of the more free-roaming nature of elk in comparison to bison as well as their shared environment, the elk reservoirs were deemed a threat to future eradication. Through a test-and-slaughter program, all known infected elk herds were eliminated and subsequently purged brucellosis from that park. The programs enacted were in line with what has been endorsed by the relevant health organizations such as the World Health Organization (WHO), the Food and Agricultural Organization (FAO), and the World Organization for Animal Health (OIE)(63). However, there exist wildlife brucellosis reservoirs of bison in eradicated countries such as Canada's Wood Buffalo National Park, with approximately 30% of the bison infected(64).

The presence of wildlife reservoirs increases the risk of outbreaks and as such requires greater vigilance because eradication within these populations is difficult to ascertain. There have been documented outbreaks originating from wildlife reservoirs, including one of bovine tuberculosis in the United States, which grew to encompass both bison and cattle herds in 20 different states(52). As both

urban and rural developments begin to further expand into wildlife habitats, the likelihood of contact between these reservoirs and domestic herds increases and the potential for a similar outbreak rises. With greater contact comes greater risk of interspecies transmission, which has been shown to be possible for *B. abortus* from cattle to humans. With the high prevalence of disease in that Wood Buffalo National Park herd but knowing that they are park-confined, the reservoir was maintained in order to reduce impact on the overall ecosystem. In addition to the benefit of the northern location of the park serving as a buffer between the reservoir and domestic herds, regular testing is also common in the region(60).

### 2.5.4 Bovine Surveillance System

The Fiji outbreak was in large part blamed upon a lack of active monitoring. A deeper look into the monitoring system and interviews with members involved determined that a deficiency of funds was largely viewed as the biggest impediment to successful monitoring(65), as they lead to a shortage of necessary professionals such as veterinarians as well as an inability to meet technical needs.

Canada finds itself in a delicate balance in maintaining eradication, with the presence of active reservoirs and its proximity to countries where the bacterial pathogen can still be found in wildlife, such as the United States, where as recently as 2010 there were over a hundred reported cases(66). In order to help minimize the spread of the disease, early diagnosis must be made. The increased development of agricultural practices in the north, paired with the implementation of other bison herds nearby, deepens the threat of a *B. abortus* resurgence. Canada developed an active monitoring system called the Bovine Surveillance System (BSS) supported by the Canadian Food Inspection Agency. The BSS involves the testing of imports as well as the routine collection of random samples from slaughterhouse cattle for serological testing, leading to the testing of more than 3000 samples annually(67). A positive sample triggers an investigation into the animal, its movements, and may lead to quarantine and potential destruction of exposed animals.

While testing was economically beneficial, it was scaled down once eradication was achieved, clearly indicating that monitoring still comes at a cost. Because the surveillance is highly dependent on the accuracy of the tests being employed, tests must be improved in order to maintain eradication, given the bacterium's ability to circumvent detection using traditional methods.

## 2.6 Pathogenicity

### 2.6.1 Pathogenic Mechanisms

While the mechanism of pathogenicity in *B. abortus* is widely debated, many agree that the bacterium's first target are professional phagocytes(68). While they are supposed to protect against infection by ingesting foreign entities, *B. abortus* is capable of surviving within these cells and uses them as a protection mechanism against the rest of the body. Upon invading the macrophage, it remains in the

vacuole and finally fuses with the endoplasmic reticulum's cisternae, where it is capable of surviving and ultimately multiplying(44). This pathogenic pathway is confirmed by the attenuated vaccine strain. The S19 live vaccine is capable of invading cells but is unable to further cause damage by multiplying as it degrades after phagosomal fusion occurs(68).

In manipulating the phagosome, *B. abortus* is able to proliferate but also evades intracellular killing. This tactic of avoiding lysosomal fusion to survive and multiply is echoed in other pathogenic bacteria, such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*(68). Despite this foundation of knowledge, the complete mechanism by which the bacterium is able to survive is not known or well-explained(24).

Overall, *B. abortus'* symptoms are a reflection of its invasion of the body, starting with the blood stream before multiplying and spreading throughout the body(69). For example, the proliferation of the brucellae brings a surge in inflammatory cells, eventually leading to vasculitis and necrosis of the surrounding tissue(70). This build up in the uterus and the ensuing placentitis consequently interferes with the metabolic relationship between an infected mother and its fetus, resulting in an abortion.

The pathogenicity of a bacterium is in large part due to the expression of certain factors, be it proteins or bacterial cell structures. As such, loss of certain genes in virulent strains leads to attenuated strains. S19, a spontaneously attenuated virus used as a vaccine, is missing a gene involved in erythritol metabolism due to a deletion that lead to a fused polypeptide(71) The lack of the most common virulence factors such as flagellum or specific secretion systems seen in other bacteria suggests that the virulence of *B. abortus* is the result of a more complex system. More specifically, virulence may stem from a coordinated multicomponent system that is involved in intracellular adaptation. Its ability to reversibly remodel the cell envelope makes it difficult to determine specific virulence mechanism as each stage likely exposes different antigenic targets(44).

Upon entering macrophages, brucellae are capable of altering intracellular trafficking as well as the intracellular environment itself, allowing for the evasion of host defence mechanisms(42).

### 2.6.2 Lipopolysaccharide

The general consensus for *B. abortus* virulence has been that S-LPS is a major component related to survival and replication and as such has become the major target of serological testing(8). Numerous attenuated strains have demonstrated an LPS structural defect, substantiating the claims about its role in virulence. Because it is such a large part of the outer membrane (OM), comprising 35-45%, the S-LPS greatly protects the cell from the external environment by contributing to the OM's structural integrity(72). The structure of S-LPS is composed of hydrophobic lipid A chains that anchors the inner and outer core of oligosaccharides to the membrane, followed by the outermost domain consisting of a repetitive glycan polymer known as the hydrophilic O polysaccharide chain.

Although the LPS seems to be involved in *B. abortus*' pathogenicity, its precise mechanism is not completely defined. It has been experimentally shown to be involved in invasion and intracellular multiplication as well as countering complement-mediated lysis(7) due to the presence of the outermost O-chain. It has demonstrated structures and properties that echo those seen in enterobacterial LPSs which exhibit low endotoxicity. Furthermore, these LPSs are highly resistant to macrophage degradation and offer protection against the immune response, much like *B. abortus*(8). However, there are differences in the type of backbone to the type of bonds linking the lipid A chains to the core(8). Because of these differences, researchers have determined that brucellae are 700 times less toxic than *E. coli* containing structurally similar LPS (8).

The O-chain is significant to virulence, having been linked to several key mechanisms including preventing complement-mediated lysis and inhibition of phagosome-lysosome fusion. This relationship was drawn from observations of the strains with a rough LPS which lack the O-chain component as these strains exhibit generally lowered virulence(73). The O-chain, through its length, acts as a shield to inhibit complement deposition, resulting in low activation of the complement pathway for S-LPS strains(8). A similar protective mechanism is demonstrated by *Salmonella spp*. and *Yersinia entercolitica*(8). Complement is the only source of opsonic protein molecules, which normally enhances phagocytosis. LPS' inhibition of complement activation leads to increased likelihood of bacterial survival(72). The steric hindrance of the mere presence of the O-chains may also innately act as a protective barrier.

The intracellular survival of the brucellae is seemingly reliant on its ability to prevent phagosome-lysosome fusion, a method exhibited by other pathogenic bacteria such as *M. tuberculosis*(68). The O-chain of LPS is essential for the prevention as depicted in a study showing that in the first few hours of phagocytosis, S-LPS strains didn't fuse with lysosomes, yet rough strains quickly fused with lysosomes and were eliminated(73). Though this study focused only on the early period of bacterial-macrophage interaction, it confirms that the O-antigen is important in the early behaviour of the bacteria.

## 2.7 Diagnostic Methods

Despite the current *Brucella*-free status of Canada, it is important to remain vigilant and maintain testing standards due to our proximity to countries where the bacterial pathogen can still be found in wildlife, such as the United States. The inability to completely eradicate disease and the high prevalence of it in certain populations is largely associated with lack of efficient diagnostic methods, which has been noted in other bacterial diseases(74). There are numerous methods that may be employed but can differ in terms of best use. For example, the gold standard is the culture and isolation method, which gives a definitive diagnosis and is labelled as such by the OIE(75). Other areas require a quicker test in order to perform screening of large populations, particularly in order to minimize the spread of the disease. There

have been some forays into the use of molecular biology techniques such as PCR, which is slowly gaining ground in terms of acceptability, with some validation studies having been completed through the OIE. However, current screening tests are heavily based on serological tests for the detection of specific antibodies. The use of specific smooth-lipopolysaccharides as antigens for tests is the present standard for presumptive diagnosis. Amongst the more commonly used, particularly at CFIA, are the fluorescence polarization assay (FPA) and competitive enzyme-linked immunosorbent assay (C-ELISA). Both techniques are described in more detail in 2.7.3 Serological Testing Methods).

### 2.7.1 Culture Plating and Isolation

The current gold standard for identification of *B. abortus* is culture plating and subsequent microbial isolation, with high specificity but lacks sensitivity where chronic brucellosis is concerned, as frequency and timing of bacteremia is crucial to collecting culturable bacteria(76). This can be done using various sources, including blood, bone marrow, and lymph nodes.

Bacterial isolation requires nutrient-rich media, such as dextrose, tryptose or soy agar, that is complex in its make-up due to the addition of as many as six different kinds of antibiotics to improve specificity (OIE 2016). When successfully cultured on plates, the elevated and smooth colonies are generally a honey color and transparent(77).

This type of testing requires very specific techniques and devices found in certain laboratories with expensive equipment(78). For example, biphasic bottles are used to decrease contamination from subculturing. These bottles have agar slants as well as broth, with subculturing performed by tilting the bottle and allowing the inoculated broth to wash over it before returning it upright. Automated systems can help detect growth by measuring changes in $CO_2$ levels. Furthermore, level 3 biocontainments are required due to the infectious, zoonotic nature of *B. abortus*.

Even with automated systems, a 95% detection rate can take up to 7 days, making it not only hazardous but also time-consuming as the bacterium is notoriously difficult to grow(38). Incubation times of up to 4 weeks for primary isolation has been documented(79), but ordinarily results are obtained within 3-4 days, with cultures considered negative after 10 days without growth(36). The combination of these requirements may lead to further delay in obtaining test results as farms and wildlife reservoirs are often located in isolated areas. Some countries as a whole are even incapable of performing this type of test due to a lack of resources and adequate laboratories(80).

The effectiveness of isolation is dependent on the amount of viable bacteria present, affecting detection rates(76). In contrast to its enhanced ability to detect brucellae, isolation is slow and expensive to perform. Despite its specificity, the major flaw of culture and isolation is its poor sensitivity, related to the stage of infection and acute versus chronic infection. To circumvent this, additional steps such as

broth culturing are performed but significantly delays results. With the complex laboratory requirements and slow growth, identification by culture is not feasible for screening use.

### 2.7.2 Polymerase Chain Reactions (PCR)

*Brucella* species are highly homologous, leading to some scientists theorizing that they are all strains of a single species. However, sufficient polymorphisms exist that the theory was refuted, and it is upon this basis that PCR assays were developed for species identification. In fact, it also recently been demonstrated to have the potential to biotype isolates, allowing for traceability in the case of outbreaks(77). For protecting the food supply and diagnosing brucellosis in humans, identifying the *Brucella* genus as a whole has become more important than identifying a species. Commonly accepted tests have been those targeting the *BCSP31* gene and the 16S-23S rRNA operon, both of which are highly conserved in *Brucella*. In fact, the 16S rRNA gene was targeted by one of the first publicly shared *Brucella* diagnostic PCR assay in 1992(81), followed later on that year by the development of a PCR assay targeting the *BCSP31* gene(82).

More specific tests for species identification requires more complex PCR assay formats. Multiplex PCR is one of the favoured, as well as PCR-RFLP (Restriction Fragment Length Polymorphisms), which adds the step of digesting the resulting amplicons with restriction enzymes(83). Ideally, real-time PCR is preferred, giving faster results as well as limiting the likelihood of misinterpretation on gels should products not be well separated after electrophoresis.

PCR as a whole is generally known to be sensitive and specific when properly optimized. Paired with its fairly inexpensive nature, it seems to offer a promising direction. However, the need for an intact *Brucella abortus* genome to act as a starting template for primers to bind to may raise an issue as the stages of infection vary and correspondingly, so do the bacterial population and where they may be located(84). As a result, there are concerns related to how long and during what periods of infection amplifiable DNA is present.

One of the advantages of PCR is its ability to analyze different specimens in which the bacterium is no longer pathogenic, offering a safer way to run tests(21). Conversely, there have been studies where serological assays have shown that the animal was infected, while simultaneous testing with PCR using blood samples revealed a negative result(84–86). However, when using milk samples and lymph tissue, the results were slightly more favourable. Similar studies have raised questions on the specimens that can be tested by PCR, as whole blood has now been identified as one of the poor specimens for PCR. With time, the use of clinical samples for testing has been improved. This advancement has come with changes in sample collection type as well as at the cost of extensive sample preparation, requiring the removal of PCR inhibitors from the matrix in order to lower limits of detection(81). Colony PCR has potential due to

the removal of an inhibitory matrix but would still require the slow step of growing and isolating colonies.

PCR can be used to identify the bacterium but does not yet seem to have the documented sensitivity and specificity seen with other diagnostic tests(36). Without proper validation studies and studies in reproducibility in these areas, it has not yet been accepted as an approved standalone method by the OIE. Instead it is relegated to the role of a complementary identification test.

### 2.7.3 Serological Testing Methods

Serological tests have been proven to be a simple inexpensive alternative with more rapid results(6), leading to their recommendation for large-scale surveillance despite the subjective nature of agglutination tests, as results must be interpreted by the technician analyzing the tests. These tests involve the use of inactivated bacterium or purified cell fractions as antigens for testing the reactivity with serum antibodies in the infected host(77). Serological tests for antibodies to S-LPS are widely used in the standard tests for the presumptive identification of infected animals.

Amongst the more commonly used serological tests are serum agglutination, Rose Bengal, and complement fixation tests. These tests cannot be considered adequate stand-alone assessments because of their low specificity. Furthermore, the lack of a standardized reference antigen can lead to discrepancies between results(87). Other tests such as the fluorescence polarization assay (FPA) have good specificity but comparatively lower sensitivity and so are recommended as screening or confirmatory tests but can be used as a standalone test if no others are available(9). FPA in particular is the test employed by CFIA and is involved in Canada's brucellosis surveillance program.

False-negative results are complicated by conditions such as subclinical infections. The prozone effect, which is an interfering immune phenomenon, inhibits the antigen-antibody interaction, as do blocking antibodies. In this case, agglutination is prevented due to excessive amounts of antibodies, which bind so completely to the antigens that the large bridged complexes cannot be formed(48). Testing during the incubation period of the brucellae and early onset of the disease often returns false-negative results due to the delay in antibody generation, similar to the lack of antibodies that may be present in chronic cases and relapse of the disease(42). As a result, serological tests must often be repeated, particularly when there is high suspicion of infection(88).

Compared to *Brucella* LPS, identical or similar O-antigens can be found in bacteria such as *Y. entercolitica* O:9 and *E. coli* O157(10, 89), leading to cross-reactivity and false positive serological results (FPSR). With the increasing rate of infection in shared reservoirs, exhibited by *Y. enterocolitica*(11) consequently raising the rate of FPSR(12), a pressing need for tests with greater specificity has been demonstrated. Similarly, the S19-vaccinated cattle elicit the same antibody response

to the O-antigen as the pathogenic strains do, further complicating the results in the identification of diseased cattle(13).

#### *2.7.3.1* Fluorescence Polarization Assay (FPA)

FPA is a relatively simple and rapid fluid phase serological test which can be used to detect antibodies to *B. abortus*. Because it can be completed within minutes, the test has been highly recommended for use in monitoring, as seen in its application in the BSS. It is technically simplistic in terms of steps with only a one-step sample dilution, measurement of background fluorescence, and the addition of the fluorescein-labelled antigen which is followed by subsequent measurement of antibody interaction(90). Samples are mainly serum but the test has been successfully performed with whole blood and milk samples(77). The test gives highly reproducible results across different laboratories(54), making it ideal for large-scale surveillance in terms of diagnosis.

The main principle of this test is based on the rotation of molecules. A fluorescent dye is covalently attached to an antigen, which is a fragment of the O-antigen in this case, and excited by plane-polarized light. Rotational differences can identify changes in molecular size, as the rate of rotation is inversely proportional to its size(90). Thus, when an antibody-containing sample is added, antibodies bind to antigens to form larger complexes, leading to the decrease in the molecular rotation and the increase in fluorescence polarization(91). Unlike other tests where analysts are required to interpret the results, FPA results are objective due to the rotational measurements involved, available two minutes after combining the antigen and the antibodies(92). Furthermore, FPA is not susceptible to the prozone effect unlike agglutination tests as it is not dependent on cross-linking.

FPA's sensitivity and specificity are dependent on the cut-off values input for negative samples. In this manner, the test can be used as either the primary test or the confirmatory test: with increased sensitivity comes decreased specificity, which would be ideal for a primary test, as false negatives could then be eliminated using a secondary test that is more specific. For example, testing has been done with a specificity of 96.6 to 98.9% depending on population factors and sensitivity upwards of 99%(93). The high sensitivity is related to the lack of washing and blocking steps, subsequently reducing the likelihood of diluting out low antibody-containing samples(94). This issue is seen in competitive enzyme-linked immunosorbent assays (cELISAs), which is a popular serodiagnostic test used worldwide. With the cut-off values chosen by the OIE, FPA improves upon cELISA while maintaining a similar diagnostic sensitivity and specificity(36). It has been noted that low, declining titres may impact the rate of detection(90).

An examination into the protocol by the United States Department of Agriculture (USDA) sought to determine the viability of the test, particularly against OIE's guidelines for the validation of diagnostic assays(54). Over ten years, the USDA's Animal and Plant Health Inspection Service (APHIS) studied the

test in order to approve it as an official test. In the end, researchers deemed it more than suitable, noting that it outperformed other already-approved tests(54). As FPA has been used in other areas of detection and monitoring outside the scope of food safety, results are considered accurate and credible. By 2009, the OIE had added the test to its manual of diagnostic tests(36) and endorsed its use as a screening test for herds and individual bovines(36).

However, because measurement requires more specialized equipment(95), this test is generally used as a preliminary evaluation, with seroreactive samples further analyzed by cELISA when possible.

#### *2.7.3.2* Competitive Enzyme-Linked Immunosorbent Assay (cELISA)

To obtain further accuracy of serodiagnosis, the samples are also tested via cELISA. This primary binding assay has a sensitivity between 92-100% and a specificity of 90 to 99%(77). Most significant is the test's ability to differentiate between antibodies acquired through vaccine or true infection due to the vaccine's low-affinity antibodies (96) and the elimination of some of the FPSR that occur in other tests(36). This is based on the competitive aspect of the test, as vaccinal antibodies are theorized to have a lower affinity for the antigens used in the test due to shorter exposure(80).

In cELISAs, S-LPS antigens are coated in 96-well plates and tested with serum. Should the suspected cattle be infected, the serum would contain antibodies that will then bind to the S-LPS antigens. To increase the assay specificity, a mAb that is specific for a common epitope of the S-LPS is used in order to compete with the sample antibody(90). By using this mAb, cross-reactions are reduced. As a result, the test is highly dependent on the sensitivity and specificity of the mAb itself(36).

An advantage for the use of cELISA in wild species is the ability to obtain good results even when using poor quality serum, including those that have been haemolyzed(36). While only taking approximately 90 minutes to run this test, the high specificity and sensitivity of the test comes at a cost that eliminates them from field use due to the numerous steps and the need for particular equipment(96). Furthermore, cELISAs historically suffer from high background noise. Washing and blocking steps were introduced in order to reduce the non-specific binding that causes that issue, at the cost of reducing sensitivity by diluting the sample. This is emphasized by the reduction in sensitivity when compared to indirect enzyme immunoassay(97).

## 2.8 Antigenic Proteins

Some diagnostic tests for other bacteria use virulence factors as biomarkers, allowing for the differentiation of vaccinated hosts from infected hosts. For example, there is a diagnostic test designed for *Mycobacterium tuberculosis* that targets lipoarabinomannan, which is involved in macrophage invasion(98) Many attenuated strains lose their pathogenicity because of the loss of these factors. While still acting as structural components of the cell, much like some virulence factors are, cell wall proteins do not usually act as identifiable virulence factors(15). They are also strongly immunogenic proteins, in

which case they can elicit an immune response. On the other hand, they may also simply be antigenic, where the proteins are simply recognized and bound to by immune system products such as antibodies or T-cell receptors, rather than stimulating the response(99). For diagnostic tests, either of these classifications can act as candidates.

Researchers have developed five approaches to determine the antigenicity of proteins. Most (4 out of 5) are heavily based on immunochemistry, while the fifth is based more on the localization of the antigenic sites(100). Because those located extracellularly are among the first proteins in contact with the host, these proteins tend to play an important role in eliciting an immune response. Their interactions with the host can lead to the modification of the host cell environment and mediate the interactions between the host cell and bacteria, which can result in antibiotic resistance(101). Localization has been deemed an important characteristic in part due to their role as the first interactors with the host.

Two other aspects of the antigenicity approach involves studying conformation and conformational changes of the protein. This is in large part due to the conformation of these proteins, which is instrumental to their antigenicity. What makes the proteins a target for antibodies are not their sequences or structures as a whole but instead certain antigenic sites, specific areas of the protein where binding of the antibody can take place(99). Should alteration of the protein's final structure occur, the required antigenic sites may become hidden and become subsequently undetectable to antibodies(102). This inhibition can be the result of becoming topographically hidden by the folding or even due to steric hindrance from surrounding structures while remaining surface-exposed.

Antigenic proteins have been used as targets for serological diagnostics, although with limited success for *B. abortus*(97).

### 2.8.1 Role in Testing

Antigenic proteins have been used in diagnostic tests for numerous other viruses and bacteria, particularly in serological tests over the past few decades(15). For example, several commercial serological kits for detecting bovine viral diarrhea virus (BVDV) use protein antigens such as $E^{RNS}$ glycoprotein(103).

The use of protein antigens in diagnostics is paired with an abundance of cautiousness due to the variability associated with disease and its stages. While there has been experimental evidence that a single antigenic protein can and has been deployed as a diagnostic tool(104) for certain bacteria, there is a general consensus that a combination of antigens is generally required in order to optimize the specificity and sensitivity of the assay over varying population(105–107). With a desire to create a pool of antigens to be used, novel antigenic proteins remain a topic of interest.

Some of *B. abortus'* outer membrane proteins (OMPs) have been explored for serodiagnostic use due to their surface exposure and role in virulence(15). There has been exploratory use of OMPs for

diagnostic testing, which have shown potential but still require further analyses and comparison(108, 109). Because the effectiveness of serological tests depends on the antigens being used, the successful identification of the antigen candidates is crucial for the assay development.

### 2.8.2 Identification of Potential Candidates

Bioinformatics is a field that uses mathematical and statistical techniques in order to interpret biological data such as genome sequences without performing further wet laboratory work. Bioinformatics strives to examine experimentally established data to find patterns and more efficiently and accurately characterize proteins based on the amino acid sequences. These efforts have led to the development of bioinformatics tools that are now able to predict the localization for those proteins that may not be easily obtained through the previously used extraction methods. Comparison of the composition, motifs, and sorting signals to similar well-defined proteins allows for identification of likely localizations of the unknown (17).

By combining several algorithmic features, the program analyzes the data given and subsequently gives a probability value to each of the five localization sites: cytoplasmic, cytoplasmic membrane, periplasm, outer membrane, and extracellular. Some proteins can be classified as having unknown localizations because their information did not give the PSORTb algorithm substantial evidence to prove they are present in any particular location. The lack of information does not exclude them as potentially being located extracellularly or in the outer membrane. In general, these bioinformatics tools can be applied to help identify antigenic candidates based on localization.

### 2.8.3 *B. abortus* Protein Antigens

The research into the use of antigenic proteins as a *B. abortus* diagnostic antigen stems from the lack of common virulence factors. Its ability to avoid activating the immune system is thus suspected to be the result of proteins influencing the cell-mediated response(15).

OMPs are one such group of proteins, with some researchers naming them as major immunoreactive components(110). Furthermore, they maintain the membrane with their structure and are responsible for its selective permeability(111). Their role in maintaining the membrane's integrity also helps them remain conserved within the genome. They are divided into three groups based on their molecular weights: Group 1 ranges from 25 to 30 kDa, Group 2 from 41 to 43 kDa, and finally Group 3 at 94 kDa(112).

The *BP26* gene, also known as *OMP28*, codes for a 26-kDa periplasmic protein that has been identified as having the antigenic potential(113). It has been experimentally shown to be able to discriminate between different stages of infection: vaccinated versus unvaccinated, as well as active versus chronic(114). Some preliminary application of the protein into an ELISA study demonstrated this protein as a potentially suitable candidate for serological detection(115). Initial Western blots seemed to

show antigenic potential(97), but another study determined that it was too specific for overall *Brucella spp*. detection across the species as well as different hosts. For example, *B. melitensis* was found in both sheep and goats, but only the sheep sera reacted with *BP26*(116). However, researchers in Brazil tested its usability in a Western blot. By following OIE guidelines for standardization, which involves analytical and diagnostic characterization, they were able to identify it as an antigen for a potential confirmatory test, although validation is still required(117). Even so, the usability of *BP26* for ELISAs is questionable, particularly when several publications have raised questions about its poor interaction with *B. abortus*-infected cattle sera(116).

*BCSP31* has been shown to possess strong immunogenicity(118). A pseudo-vaccine using the gene underwent preliminary testing in rabbits, resulting in an induction of antibody titres(119). Its specificity is well-documented, with BLAST analysis performed to confirm the lack of homology with other species such as *Yersinia spp*. and *Salmonella spp*.(83). In fact, PCR techniques have been developed for the *BCSP31* genes. One such method has the *BCSP31* gene paired with three other genes (OMP2B, *OMP2A*, and OMP31), thus allowing for the identification of *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis*(120). Yet another combines the gene with six others in order to identify six species(120). Despite its success as a target for PCR due to its highly conserved nature, no further work has been published in regard to its use in serodiagnosis.

The principle of using protein antigens in assays is not a novel one, but has been employed with limited success, particularly with *B. abortus*. Based on reviewing the OIE's 2016 Terrestrial Manual, there is no direct mention of a particular antigenic protein employed.

# 3. MATERIALS AND METHODS

## 3.1 Selection of Protein Candidates

### 3.1.1 *Bioinformatics Tools*

The genome of *B. abortus* contains more than 3000 ORFs(32). Bioinformatics tools were employed to analyze these ORFs for the identification of the encoded proteins with diagnostic potential based on protein subcellular localization. Proteins in the extracellular space and outer membrane were selected due to their exposure to the immune system; previous studies have shown that antigens are favourably formed on surface areas that are more exposed(14). This has been noted in bacteria with similar cyclical fever symptoms such as *Borrelia burgdorferi*, where surface-exposed proteins are the target for antibodies and subsequently act as candidates for vaccine development(121).

PSORTb v3.0.2 is a subcellular localization prediction tool used in the present study. It uses a combination of algorithms with a focus on high precision predictions, leading to higher specificity (122).

This program combines the three aforementioned approaches. These algorithms are initially based on amino acid sequences. PSORT also has its own database, known as PSORTdb 3.0, which contains genomes precomputed through both laboratory work and computational predictions.

### 3.1.2 *Candidate Selection Criteria*

Whole genome sequences of different *B. abortus* strains are available from a public database (NCBI Genome). They were analyzed using a subcellular localization tool (PSORTb v3.0) to generate information on protein subcellular localization. Candidates were selected based on two major criteria; 1) that they are those located extracellularly or in the outer membrane and 2) are conserved across numerous strains. Other antigenic proteins reported in literature and/or identified by previous proteomics studies were added to the candidate list for immunological analysis.

### 3.1.3 *Protein Candidates Encoded by Genomes of B. abortus strains*

Using the Genome Assembly and Annotation report tab of the *B. abortus* page from NCBI Genome (https://www.ncbi.nlm.nih.gov/genome/genomes/520?), the sequence information was limited to only those strains with the complete genome. At the time of research, NCBI Genome had thirteen fully sequenced strains, which were downloaded and submitted to PSORTb. The resulting data identified all *B. abortus* proteins, organized by chromosome. Each of these files were amalgamated into a single Excel document.

By combining the results of all the strains by each chromosome and sorting the file by sequence ID, it was possible to identify proteins that were highly conserved across multiple strains. To be selected from this particular process, the protein had to be: (a) localized on the surface of *B. abortus*, (b) be present in the genomes of seven or more strains of *B. abortus,* and (c) be specifically present in strain 86/8/59. This last restriction is due to that strain's availability within CFIA for wet laboratory work.

This study mainly focuses on the conserved proteins located in the extracellular space and outer membrane. To expand the number of proteins included in this project, the list of proteins was expanded to include the highly conserved proteins of unknown subcellular localization, which meant they had to be present among at least ten of the 13 strains. Furthermore, a literature search was conducted in order to pinpoint suspected antigenic proteins identified through wet laboratory work. Several were selected and also included in the candidate pool. While these candidates were exempt from parts of the criteria, they still had to be present in strain 86/8/59.

## 3.2 Media, and Buffer

All overnight cultures were prepared in autoclaved Luria-Bertani (LB) broth. Broth was made in quantities of 500 mL (12.5 g broth powder (BD Biosciences, Sparks, MD, USA), 500 mL Milli-Q water). The broth was thoroughly mixed to ensure the LB broth powder was dissolved and immediately

autoclaved at 121°C for 20 minutes. The broth was good for use up to 6 months after date of manufacturing. When required for selective growth, the broth was supplemented with 100 µg/mL of carbenicillin.

Similarly, LB agar was used for growth of isolated colonies, with 100 µg/mL of carbenicillin used in order to limit growth only to those containing pIVEX vectors, although further screening was used to confirm.

## 3.3 Cloning

### 3.3.1 *Cloning Strategies*

For gene cloning, two techniques can be used: *in vitro* or *in vivo*. *In vitro* cloning is simply the creation of copies of fragments of DNA by PCR. For the purposes of this study, the *in vivo* gene cloning method is chosen as it is a rapid method that allows for cloning of a target gene into a plasmid vector through homologous recombination *in vivo* in *E. coli*. This *in vivo* cloning technique is in contrast to the conventional cloning technique that relies on restriction endonucleases to digest both the target gene and the plasmid vector as well as T4 DNA ligase to combine the target gene with a linearized plasmid vector *in vitro*. Instead, this modified *in vivo* cloning uses PCR to generate a linearized vector and a target ORF sequence with homologous ends and takes advantage of cloning within *E. coli* cells through homologous recombination. Co-transformation of *E. coli* with the two PCR products will result in a recombinant plasmid *in vivo*.

### 3.3.2 Vectors

There is a wide variety of vectors available for cloning, dependent on the downstream application of the recombinant plasmids. In this study, the purpose of cloning an ORF into a plasmid vector is to create an expression construct that allows for the generation of a recombinant protein in cell-free expression system based on *E. coli*. A pIVEX2.3d vector (Figure 3) was chosen for cloning and used in conjunction with Roche's cell-free RTS *E. coli* system (Roche Diagnostics) for *in vitro* transcription/translation. The recombinant protein thus produced contains a 6×His-tag at the C-terminus.

For those candidates that failed to express using pIVEX2.3d, the process was repeated using a pIVEX2.4d vector (Figure 4) for cloning. The resulting recombinant protein contains a 6×His-tag at the N-terminus. These His-tags are necessary for the detection of protein formation and expression using a Western blot with anti-His antibodies.

From a frozen glycerol stock stored at -80°C, an inoculating loop was used to streak a carbenicillin-(100 µg/mL) supplemented LB agar plate for pure colonies and incubated at 37°C overnight. LB broth (5 mL broth + 5 µL of carbenicillin) was inoculated with the vector and shaken at 250 RPM

overnight at 37°C (MaxQ 4000 Incubator Shaker, Thermo Barnstead). To complete preparation of the vector, the plasmid was isolated using a QIAprep Spin Miniprep Kit (QIAgen, Mississauga, Ontario).

##### *3.3.2.1 Linearization of Plasmid Vector*

Both vectors were digested by the *Nde*I enzyme in order to achieve a linear sequence (Figure 5b), which was achieved as per the New England Biolabs (NEB) protocol: 1 µg of DNA, 5 µL of 1x CutSmart Buffer, 1 µL of *Nde*I, with nuclease-free water to bring the reaction volume to 50 µL. Because the DNA was purified simply by a miniprep procedure prior to digestion, the reaction was incubated in the thermocycler (Eppendorf Mastercycler 5345 Gradient S) at 37°C for 60 minutes to obtain complete digestion. The thermocycler program included a heat-inactivation step, performed at 65°C for 20 minutes.

The enzyme-digested vectors underwent PCR amplification in order to eliminate the multiple cloning site (Figure 5B). PCR also eliminates the overhangs from the multiple cloning site and subsequently creating direct targets for homologous recombination (Figure 5b). These primers are listed in Table 2. Elimination of these overhangs decreases the likelihood of vector recircularization. The PCR was performed with a reaction volume of 50 µL. The PCR parameters included an initial denaturation at 98°C for 30 seconds. The cycling parameters of the protocol had a 10 second denaturation at 98°C, a 30 second annealing stage at 55°C, and elongation at 72°C for 2 minutes. These 3 stages repeated for a total of 30 cycles. A final 10-minute elongation at 72°C completed the reaction. Reactions were held at 4°C until the product was cleaned up.

The products were isolated via using a QIAgen QIAquick Gel Extraction kit (QIAgen, Mississauga, Ontario) from a 1% agarose gel. Bands were visualized with a handheld UV light (UVGL-58 Handheld UV Lamp, UVP) and cut using a scalpel. Two tubes of the same sample were run through one column in order to concentrate the sample. This isolation allows for the removal of any uncut vector, which would appear as secondary bands. Concentration of the final product was measured through NanoPhotometer® (P330, Implen), which was blanked with the same Buffer EB from the kit.

Co-transformation of *E. coli* with the linearized vector and an amplified ORF allows homologous recombination to occur *in vivo*, resulting in a circular plasmid with the target insert (Figure 5C). This entire process is demonstrated in Figure 5D.

### *3.3.3* Preparation of Genomic DNA from *B. abortus*

*B. abortus* strain 86/8/59 was heat inactivated by Dara Lloyd at CFIA’s Ottawa Laboratory Fallowfield (OLF). The strain was first isolated in 1959 by a laboratory in Weybridge, England, from a bovine fetus(123) and serves as one of the reference strains. Two other strains were also heat-inactivated and available for use. These three strains are listed in Table 1.

DNeasy Blood & Tissue kits (QIAgen, Mississauga, Ontario) were used to extract DNA from the cells following the manual with the spin-column protocol. Extracted material was further aliquoted, with 5 $\mu$L in each 0.2 mL tube to reduce freeze-thaw cycles over time and use.

*Table 1. Working strains available in the lab for use, with NCBI Genome assembly status.*

| Strain ID | Chromosome | Accession Number | Assembly Status |
|---|---|---|---|
| 86/8/59 | 1 | NZ_CP007751.1 | Complete |
| | 2 | NZ_CP007764.1 | Complete |
| S19 | 1 | NC_0101742.1 | Complete |
| | 2 | NC_0101740.1 | Complete |
| 544 | - | - | Scaffold |

### 3.3.4 Amplification of ORFs by PCR

In order to achieve co-transformation, both the ORFs and the enzyme-digested pIVEX2.3d were amplified by PCR (Figure 5A and B). Successful isolation of ORFs from the genomic DNA was determined by agarose gel electrophoresis.

The enzyme-digested vector underwent PCR amplification, creating target ends for cloning. Formation of recombinants was further encouraged by the PCR primers created for each individual ORF, which added extra base pairs complementary to the vector's target ends. The elimination of PCR by-products by way of gel extraction ensured that recombination was limited to the desired ORF and vector.

Primers for each selected ORF were specifically designed in order to encourage homologous recombination, as demonstrated in Table 3. One end of the primer was the complement to the end of the ORF, represented by the red letters of the primer designed in Table 3. The black letters of the primer represent an additional segment of 25 or 26 nucleotides that were homologous to the ends of the PCR product of the linearized vector, with 25 being added to the reverse primer and 26 to the forward. Primers designed for use with pIVEX2.3d and pIVEX2.4d are described in Table 4 and Table 5 respectively.

The thermocycler (Eppendorf Mastercycler 5345 Gradient S) ran at 98°C for 30 seconds initialize the PCR reaction. Subsequently, the following cycling protocol of denaturation, annealing, and elongation was run for 30 cycles. Denaturation of double-stranded DNA (dsDNA) was achieved by running the cycler at 98°C for 10 seconds, followed by 30 seconds at 56°C in order for annealing to occur. Elongation was performed at 72°C, although the length of time varied per sequence length at 90 seconds/kb based on length of target. Final extension was performed at 72°C for 10 minutes.

### 3.3.5 Transformation of *E. coli* DH5α

*Escherichia coli* cells (DH5α) glycerol stocks were kept at -80°C. Using a 1 µL inoculating loop, a single loop of the cells was streaked on an LB plate containing no antibiotics and incubated at 37°C

overnight (Thermo Scientific™ Heratherm™ General Protocol Oven). With a new loop, a single colony was inoculated into 5 mL of LB broth and put in the MaxQ 4000 Incubator Shaker (Thermo Barnstead) at 200 rpm overnight at 37°C.

With 400 µL of the culture, a 1/100 dilution subculture was made in 40 mL of LB broth. The subculture was incubated at 37°C while agitating at 200 rpm until the $OD_{600}$ was approximately 0.35 ($2.8 \times 10^8$), with the OD measured every 20 minutes after the first 2 hours of incubation (Implen NanoPhotometer® P 330). The culture was transferred into a 50 mL Falcon tube and kept on ice for 10 minutes, after which it was spun down at 2700 × g at 4°C for 10 minutes. After discarding the supernatant, the pellet was resuspended in 24 mL of ice-cold filter-sterilized 80 mM MgCl2- 20 mM CaCl2 by placing the tube horizontally in an ice tray and rotating the tray on the Belly Dancer (IBI Scientific) for about 20 minutes or when the pellet has fully dissolved. The tube was again spun at 2700 × g at 4°C for another 10 minutes, with the supernatant discarded. The pellet was resuspended in 1.6 mL of ice-cold 0.1 M CaCl2 in 10% glycerol by shaking it in an ice tray on the belly dancer, with occasional slow inversion. When completely resuspended, the bacteria suspension was dispensed in aliquots of 50 µL and stored at -80°C in screwcap tubes for future use.

Transformation of DH5α was done by heat shock with a 2:1 insert to vector ratio, using 100 ng of the vector (124). The aliquot of DH5α was thawed before the insert and vector were added, with subsequent mixing performed by pipetting slowly. The reaction tube was incubated on ice for 30 minutes before the cells were heat shocked in a 42°C dry bath for 30-45 seconds. The tube was then returned to the ice tray for 2 minutes.

After adding 250 $\mu$L of room temperature LB broth to the tube, it was incubated on the shaker at 37°C (250 rpm) for 1 hour. The entire reaction volume was plated on a carbenicillin (100 $\mu$g/mL) supplemented LB agar plate. Plates were incubated upside-down at 37°C overnight (Thermo Scientific™ Heratherm™ General Protocol Oven). Controls were included throughout the process, with undigested vector plated at a 100-fold dilution as a positive control and the digested, PCR vector product acting as the negative control. Successful transformation was determined by the growth of colonies on carbenicillin-treated LB agar, which were picked and grown overnight in carbenicillin-supplemented LB broth. This selective growth process was made possible due to the presence of the AmpR, which offers ampicillin/carbenicillin resistance.

To confirm that the colonies that were growing contained recombinant plasmids, colony PCR using Taq DNA polymerase was used to identify successful recombinant ORFs.

### 3.3.6 Confirmation of the ORFs Inserted into Plasmid Vector

#### *3.3.6.1 Colony Growth and Screening by PCR*

Not all the resulting colonies from cloning will contain the desired insert, should recircularization of the plasmid occur. As a result, molecular cloning requires screening in order to determine the successful addition of the insert.

From isolated colonies that grew on the plate, a single colony was picked with a loop and inoculated into 5 mL of LB broth. The culture was left to incubate at 37°C overnight in the shaking incubator at 250 RPM. The following day, 100 µL of the culture was centrifuged (13 000 x g for 2 minutes) in order to collect the pellet. Upon discarding the supernatant, the pellet was resuspended in 100 µL of 50 mM NaOH and vortexed for 1 minute. The suspension was heated at 100°C for 10 minutes and 10 µL of 1 M Tris-HCl (pH 8.0) and mixed. The tube was spun down (13000 x g for 1 minute) in order to isolate the supernatant.

Initialization of the PCR reaction was performed at 94°C for 4 minutes. Thirty-five cycles of the following three steps were performed: 30 seconds at 94°C, 45 seconds at 55°C and 2:20 minutes at 72°C. Final elongation was completed at 72°C for ten minutes. PCR products were run on a 1% agarose gel for 25 minutes at 120V before being visualized under UV light (Gel Doc 2000 System, Bio-Rad). Images were taken with Quantity One analysis software (Bio-Rad, Mississauga, ON). Successful recombinants were approximately 300 bp longer than empty vectors, due to the addition of the sequences that are part of the T7 promoter and terminator.

Again, PCR products for both ORFs and plasmids were gel-extracted in order to reduce the effect of primer dimers and unwanted secondary products on future transformation. The gel extraction was done as described previously with the QIAgen QIAquick Gel Extraction Kit.

Our transformation and cloning protocols were based on homologous recombination specific to each end, making switched orientation impossible. In this case, backbone primers are more beneficial for high throughput screening due to it being vector-specific rather than insert-specific. They work by annealing to sites present on the vector flanking the insert site, which in this case were the promoter and terminator sites. As a result, colony PCR can be used to identifysuccessful recombinants.

#### *3.3.6.2 Frozen Stock*

In order to keep a viable stock of the recombinant *E. coli*, 700 µL of culture was combined with 300 µL of 50% glycerol (1:1 sterile glycerol to LB broth). This stock was kept in the -80°C freezer for future use.

## 3.4 *In Vitro* Transcription and Translation

*In vitro* transcription/translation was used for protein expression. The *in vitro* environment has the advantage of eliminating the need to maintain the cell and subsequently frees up metabolic resources that

would otherwise be used to support the cell's viability (125). These extra resources can then be focused on the formation of the desired protein, ideally resulting in the expression of a single protein using a simple, quick protocol.

After running 4 mL of the overnight culture of the recombinant product from section 3.3.6.1 through QIAprep Spin Miniprep Kit, it is once again quantified using (Implen NanoPhotometer® P 330). Protein expression was completed using an RTS 100 *E. coli* HY kit (5Prime, Hamburg, Germany), with 50 μg of template per reaction. To maintain the integrity of the components, each reaction component was aliquoted upon resuspension in order to limit each aliquot to a maximum of 2 freeze-thaw cycles. After a 6-hour incubation in the thermal cycler (Mastercycler Personal, Eppendorf) at 30°C, the products were left at 4°C overnight in order to achieve full maturation of the desired protein. Success of the protocol was measured by the fluorescence of the control vector GFP included with the kit and run in parallel with the candidates. Proteins were used immediately or aliquoted in 5 μL volumes to be frozen at -20°C.

To test the performance of the transcription/translation kit, the entire experimental design starting from the PCR and transformation stage was tested with GFPuv as fluorescence would indicate successful folding without performing a Western blot.

## 3.5 Detection of Recombinant Protein Expression

Because of the presence of the N or C-terminal His-tag on the plasmid vector, successful protein formation could be assessed through Western blots with specific anti-His antibodies. In particular, those that failed to express with pIVEX2.3d were re-tested with pIVEX2.4d to offer a second opportunity for His-tag exposure.

The preliminary Western blotting used for screening was achieved using a general penta-His antibody with a secondary antibody chosen to bind to the primary antibody. Horseradish peroxidase was used as a reporter enzyme, as the secondary antibody was peroxidase-conjugated. This method non-discriminately screens for any folded protein, rather than screening for antigenicity.

In order to visualize formed proteins, 10 μL of each sample was run per lane on an SDS PAGE (4% stacking and 12% resolving gel) along with the protein standard for size comparison (Precision Plus, Bio-Rad). It was run at 200 V for 40 minutes before the separating gel was transferred onto a nitrocellulose membrane via a semi dry transfer apparatus (Trans-Blot, Bio-Rad) (15V, 30 minutes) for Western blot procedure. Immunostaining was achieved by first blocking the membrane with 3% IgG free BSA (w/v) in PBSTT for 1 hr at room temperature with agitation (Belly Dancer Shaker, IBI Scientific). Alternatively, this step was occasionally run overnight agitating at 4°C. The remaining BSA was rinsed with 1X PBS-TT, once again by agitation. The primary antibody, a penta-His mouse monoclonal antibody was used to probe the membrane while diluted 1:25 in 3% BSA/1 X PBS-TT and incubated for an hour at

room temperature with slow shaking. Removal of unbound antibody was achieved through 5 washes of the membrane with 1X PBS-TT for 3 minutes each wash. The secondary antibody solution (peroxidase-conjugated goat anti-mouse IgG (H+L) was applied (1:1000 dilution in 3% BSA/1 x PBS-TT) for an hour on the shaker at room temperature. The 5 washes were repeated, and results were developed using the HRP conjugate substrate kit (Bio-Rad) according to the supplier's instructions. A 10-minute water wash was done before the membrane was allowed to air dry.

Successful proteins were subsequently used for screening using serum with a dot blot Western method. Proteins were initially dotted directly onto a nitrocellulose membrane at 1 μL and underwent western processing with the iBind Flex Western System, with the same primary and secondary antibodies as in the initial protein formation visualization Western protocol in order to allow for standardization.

### 3.5.1 Standardization of Recombinant Proteins

Once proteins demonstrated a detectability with a non-specific Western, further information must be extrapolated in order to properly perform the final screen for antigenicity. While the *in vitro* transcription/translation generates a considerably higher amount of protein than *in vivo* methods, the final concentrations vary per reaction. For our purposes, exact quantification was not required as there was not a specific amount of protein required for this assay. Instead, the desire is to have the same quantity of protein tested per sample, which can be simply adjusted with the use of the proportional volumes of samples.

Quantity One is the program with which Western Blot images were taken, combined with the Gel Doc 2000 system. The volume tools allow for the quantification of dot blots and other arrays, measuring signal density within a chosen boundary. Using the volume toolset, the volume circle tool was used to draw a circle around each dot blot. The Volume Analysis Report button displays the information that can be displayed in the final Volume Report.

In order to reduce interference by background pixels, Quantity One has an option for background subtraction. Using the volume tool, encompass an area that has no data and appears equal to the background noise seen around the data points. When choosing Volume Properties, select the Background option, which will determine the average intensity of the pixels in the background. This value will be subtracted from each pixel in the unknown volumes and eliminate any background pixels. To enable this feature, the Volume Report Options → Background Subtraction Method must be toggled to the "Global" button. For the purposes of this study, the most important values from the volume report is the adjusted volume, which gives the volume minus the background volume.

To normalize the protein concentration tested, the highest adjusted volume is determined. This protein will be dotted at 1 μL. Subsequently, with this volume as the dividend and the other data points in

question acting as the divisor, the approximate amount in μL to be tested for every data point is determined. By comparing the relative densities of each individual protein to the reference's, individual sample volumes could be determined, achieving the end goal of performing the final screening with proteins at similar quantities.

## 3.6 Detection of Anti-*B. abortus* Antibodies in Sera

### 3.6.1 Serum

Sera isolated from *B. abortus*-infected animals should contain the antibodies produced in the presence of the specific bacteria. The antibodies in the sera can be used as primary antibodies to screen the formed proteins. Subsequent binding would indicate that an interaction occurred between the two, which would suggest the presence of antigenic proteins. Numerous sera were supplied by CFIA Ottawa Laboratory Fallowfield, of varying infectious reactivity. A series of experiments were performed on the sera themselves for a variety of purposes: (1) to test each serum's reactivity, (2) determine working dilutions, and (3) to determine if pre-adsorbing the serum with E. coli at different dilutions reduced the background reactivity, particularly with homologous proteins found in other bacteria.

#### *3.6.1.1 Reactivity of Sera*

In order to test the reactivity of the serums given to us, LPS-coated plates were provided by CFIA Ottawa Laboratory Fallowfield and kept frozen upon receipt. When ready for use, a plate was taken out from the freezer and left at room temperature. After thawing for an hour, the plate was washed with PBST using the BioTek 405 LS ELISA Plate Washer and left to incubate for an hour at room temperature. The sixteen sera were prepared by performing a serial dilution series (1/100, 1/200, 1/400, 1/800, and 1/1600) in PBS to determine working concentration for future use. Additionally, a known positive, a known partial positive, and a C++ control were diluted. Upon addition of these 19 sera and the negative control of water, the plates were incubated for an hour at room temperature. After the plate was washed with the plate washer, the secondary antibody (peroxidase-conjugated Affinipure goat anti-bovine IgG (H+L)) at a dilution of 1/1000 was added to each well. The plate was again incubated for 1 hr at room temperature. The secondary antibody was removed by the plate washer and the substrate (0.5 mL ABTS, 0.1 mL $H_20_2$ (3%), and 10 mL Citrate buffer) was added and shaken for 10 minutes (Compact Digital Microplate Shaker, ThermoScientific). The plate was read by a microplate spectrophotometer (Epoch 2, BioTek). Test sera were selected based on their reactivity at a feasible dilution level (i.e., when the ELISA reaction was not saturated).

#### *3.6.1.2 Pre-adsorption of Sera*

Prior to use in dot blot assays, a pre-adsorption method was employed, using *E. coli*, based on documented methods(126–128). The serum can be added to a stock suspension of the cross-reactive species, which is the method employed in this study with *E. coli,* where the cross-reactive bindings will

bind to the antigens presented by *E. coli*. After adsorption was performed, the *E. coli* can be isolated into a pellet, which would then also contain the cross-reactive antibodies (126). The harvested supernatant becomes the pre-adsorbed serum. Previous studies have compared the use of untreated serum vs. pre-adsorbed serum in protein blots and noted that there were proteins that only appeared when probed with the untreated serum(127). This method allows for the reduction in background noise as well as gives stronger confidence in the specificity of the serum.

The first step of the protocol required growing cultures of *E. coli* ATCC 25922 in LB broth overnight shaking at 250 RPM at 37°C (MaxQ 4000 Incubator Shaker, Thermo Barnstead). This is followed by centrifugation in order to isolate the whole cells. After being washed and resuspended, the cells were incubated with the serum before the bacteria was once again pelleted. This pellet would then consist of the *E. coli* cells as well as any antibodies from the serum that bound to these cells, which would have likely otherwise lead to cross-reactions in the dot blot. As a result of this process, the supernatant should now consist of serum with a higher specificity for *B. abortus*. Proteins were subsequently screened using different serum conditions in order to determine antigenicity.

### 3.6.2 Dot Blot Assay

Antigenicity was determined using ImageJ, an image processing program, by measuring the relative amount of protein detected relative to the positive control, LPS. As a negative control, *E. coli* lysate was used to measure the background antigen-antibody interaction.

For the first dot blot assay, performed large scale and in the same order as the initial His-tag screening, each protein was tested at the specified volumes from the pre-determined values obtained in 3.5.1 (Quantity One – Standardization). It was performed using an iBind Flex Western System with 4 different serum conditions (S+, S22, pre-adsorbed S22, and negative) acting as primary antibodies. The secondary antibody (peroxidase-conjugated Affinipure goat anti-bovine IgG (H+L)) was used at a 1/200 dilution. The results were visualized using the HRP conjugate substrate kit (Bio-Rad) and subsequently washed with water for 10 minutes while agitating.

It was anticipated that with a dot blot assay, not only could antigenic proteins be identified, but their relative antigenicity may be anticipated based on the strength of the interaction with the antibodies in the serum. The formation of antigen-antibody complexes can be correlated to the amount of protein comparatively visualized(129). This is possible as the amount of protein being assessed was already standardized across the assay. What would ordinarily be considered the quantification of the protein is now an indicator of the amount of interaction occurring between the candidate and the antibodies in the serum and thus corresponds to the antigenicity of the protein. Thus, in order to assess interaction, relative density of the samples was again measured.

In this case, *E. coli* lysate was included in the screening, acting as a negative control. In addition, it provided the baseline threshold for background antibody-antigen interaction, due to its interactions even with pre-adsorbed serum.

Each serum condition was once again analyzed with Quantity One, as previously described. Once the relative densities were obtained for each candidate and normalized against the LPS to obtain a percentage value.

Although Quantity One was previously used for the first, larger-scale screenings, the second and third dot blot assays of each sera condition were analyzed with Image J. Quantity One was incompatible with the secondary computer's operating system. Image J is an image processing program with an emphasis on scientific images with which relative concentrations can be determined (130).

In this case, the Protein Array Analyzer tool (http://image.bio.methods.free.fr/ImageJ/?Protein-Array-Analyzer-for-ImageJ.html) is an extension that can be added to aid in this analysis. A conversion of the jpg files into 8-bit was first performed, with options available for background subtraction. The default setting was used, with the "Subtract Background" option set to "Linear" and "Radius for 2D Rolling Ball subtract background" set to "25". This step also leads to an inversion of the colours. The cursor diameter was adjusted to 9. Because the dots of this assay are not perfectly aligned, each individual dot must be selected. The cursor is set to encompass the dot area without overlapping any of the horizontal or vertical lines used to mark each region. Once placed click the "Measurements > Add" button. This step is repeated for every dot area.

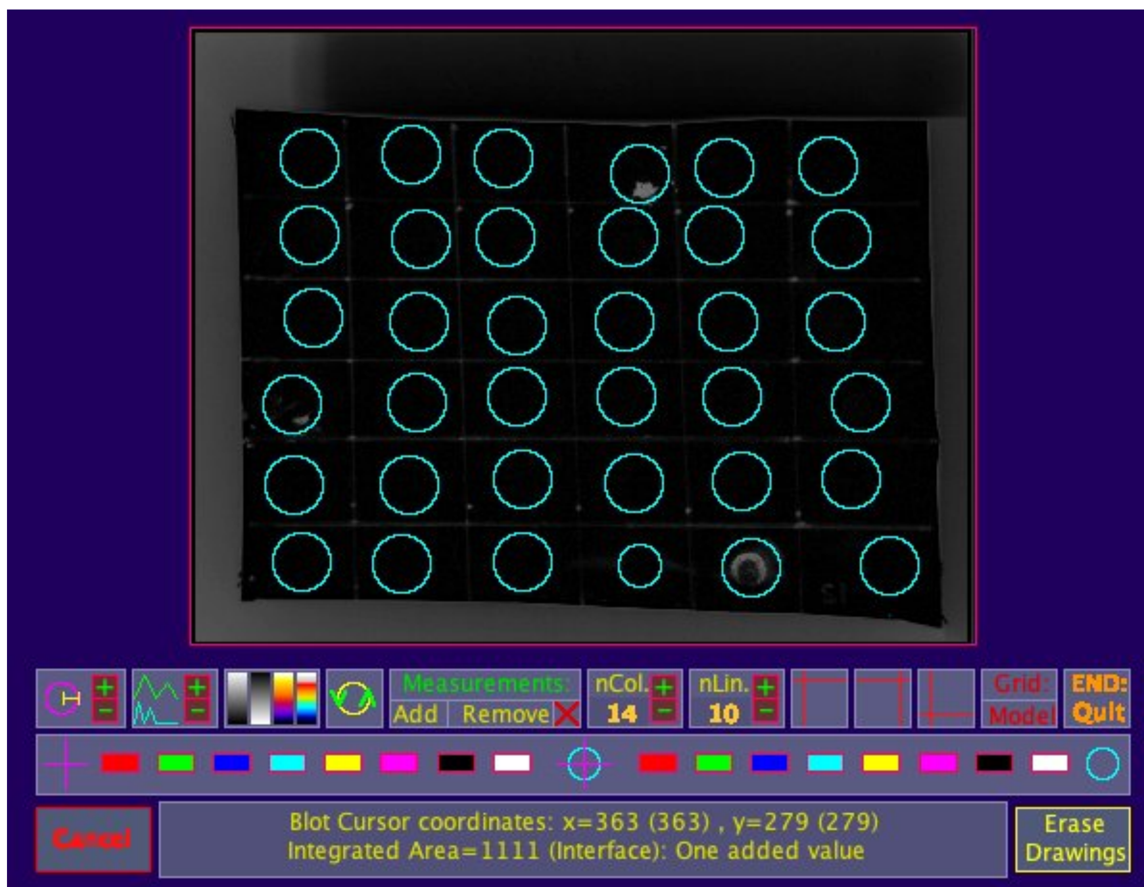


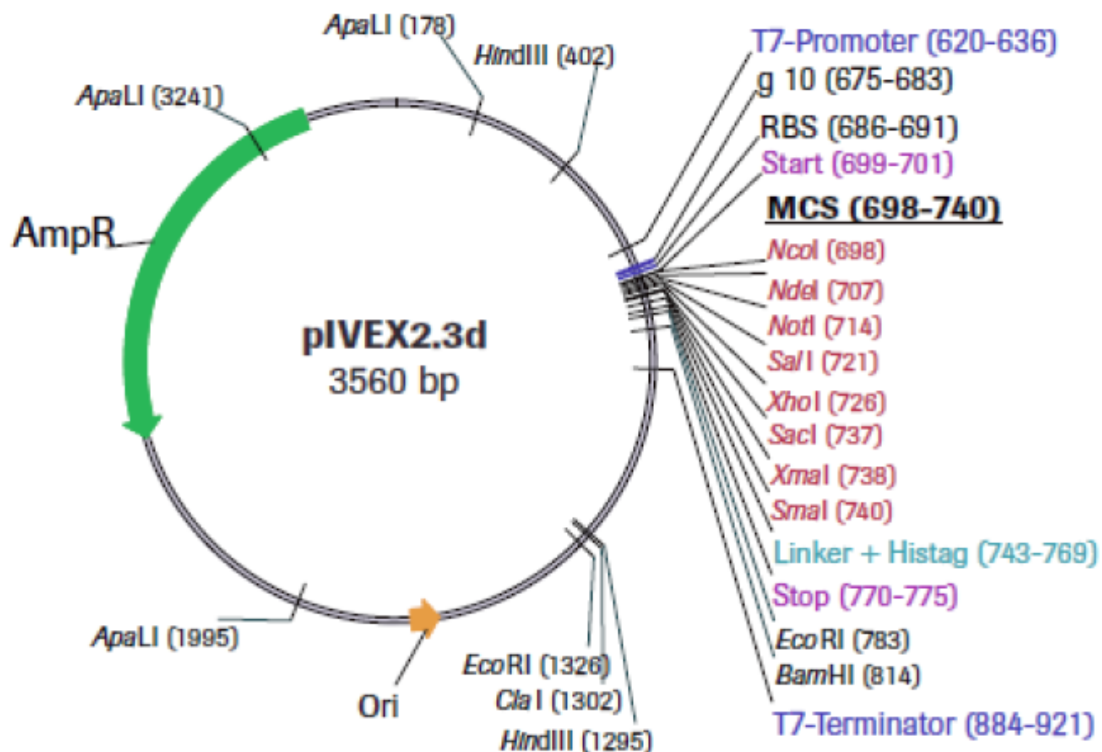

*Figure 2. Example of the Protein Array Analyzer Palette, an extension of Image J. Dots of this serum-probed dot blot assay are individually encompassed by the cursor in order to have their densities analyzed by the program.*

Measurements appear in a separate tab, with each dot identified in order upon which they were measured. The integrated area is the key number in this tab. These values are saved (Array Analysis → Save Results Documents).

The measurement results can be re-arranged into table form, to resemble the dot blot layout. The measurement obtained for the *E. coli* lysate is subtracted from every value as the background interaction. Subsequently, the interactions were relatively quantified against the LPS positive control. With LPS representing 100% antigen-antibody interaction, the background-subtracted measurements of each candidate were measured against the LPS in order to obtain a percentage of interaction.

Using this percentage value, selection for positive interactions was done. The average of all the positive relative interactions was taken for the conditions, as well as the standard deviation. Any candidates that fell outside of the limit (the average plus the standard deviation) was considered an outlier and eliminated in order to obtain a truncated average. The interaction was considered to be positive if the percentage was higher than this truncated average. The use of the truncated average was arbitrary. For further exploration, the threshold was dropped to 40% of the truncated interaction.

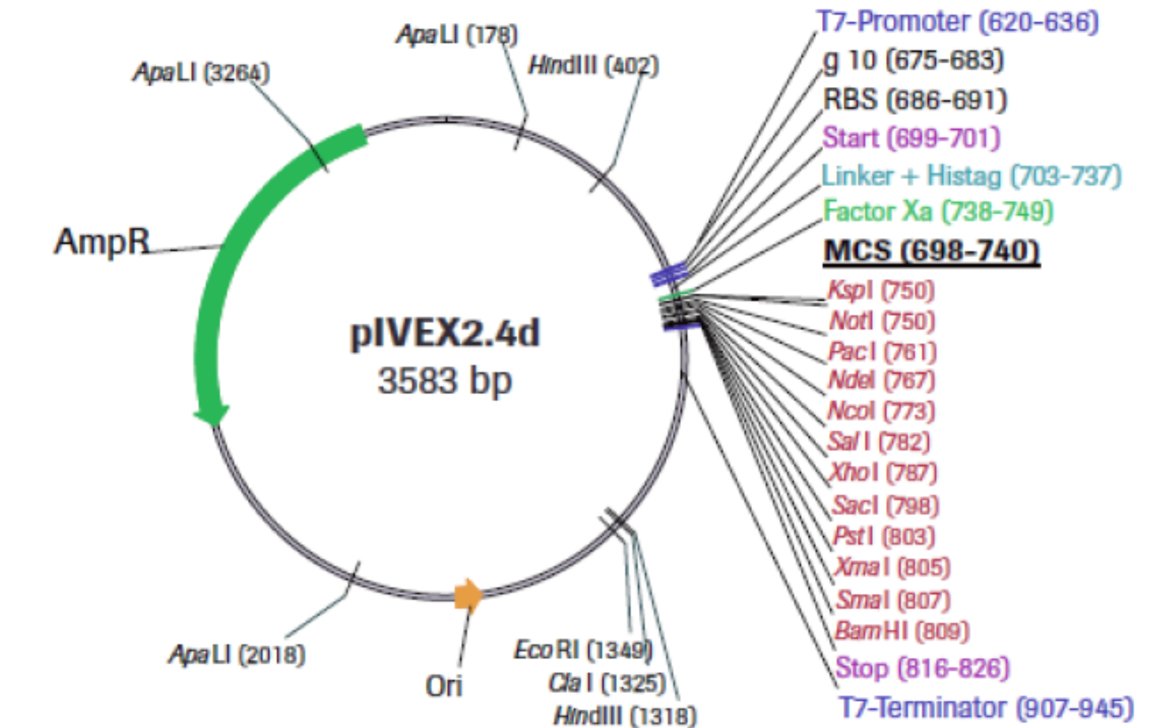
From the first initial screening, the successfully detected proteins went through two more dot blot assays, using the same method previously employed. By eliminating proteins that had already failed to display interaction, the dot blot assay was streamlined. Furthermore, the repeat testing of the promising candidates gave a total n of 3 tests for each serum. Proteins were then classified by their total number of positive interactions across the three tests for each condition. The most promising candidates would be those that were successful for all nine tests.




```
                                   T7-Promoter
601   GATCTCGATC CCGCGAAATT AATACGACTC ACTATAGGGA GACCACAACG
      CTAGAGCTAG GGCGCTTTAA TTATGCTGAG TGATATCCCT CTGGTGTTGC
                                g10 ε         RBS         NcoI
651   GTTTCCCTCT AGAAATAATT TTGTTTAACT TTAAGAAGGA GATATACCAT
      CAAAGGGAGA TCTTTATTAA AACAAATTGA AATTCTTCCT CTATATGGTA
                                                            Me
                                                 XmaI
         NdeI      NotI      SalI   XhoI     SacI  SmaI    Linker
701   GGCACATATG AGCGGCCGCG TCGACTCGAG CGAGCTCCCG GGGGGGTTC
      CCGTGTATAC TCGCCGGCGC AGCTGAGCTC GCTCGAGGGC CCCCCCAAG
      tAlaHisMet SerGlyArgV alAspSerSe rGlu           GlyGlySe

       Histag                                EcoRI
751   TCATCATCAT CATCATCATT AATAAAAGGG CGAATTCCAG CACACTGGCG
      AGTAGTAGTA GTAGTAGTAA TTATTTTCCC GCTTAAGGTC GTGTGACCGC
      rHisHisHis HisHisHis* *****
```

*Figure 3. pIVEX2.3d vector map from biotechrabbit*(131).




```
                                  T7-Promoter
601   GATCTCGATC CCGCGAAATT AATACGACTC ACTATAGGGA GACCACAACG
      CTAGAGCTAG GGCGCTTTAA TTATGCTGAG TGATATCCCT CTGGTGTTGC

                                   g10 ϵ       RBS
651   GTTTCCCTCT AGAAATAATT TTGTTTAACT TTAAGAAGGA GATATACCAT
      CAAAGGGAGA TCTTTATTAA AACAAATTGA AATTCTTCCT CTATATGGTA
                                                       Me

                                                      KspI
       Linker + Histag                          Factor Xa NotI
701   GTCTGGTTCT CATCATCATC ATCATCATAG CAGCGGCATC GAAGGCCGCG
      CAGACCAAGA GTAGTAGTAG TAGTAGTATC GTCGCCGTAG CTTCCGGCGC
      tSerGlySer HisHisHisH isHisHisSe rSerGlyIle GluGlyArgG

          PacI        NdeI      NcoI        SalI XhoI    SacI   PstI
751   GCCGCTTAAT TAAACATATG ACCATGGCAA GTCGACTCGA GCGAGCTCTG
      CGGCGAATTA ATTTGTATAC TGGTACCGTT CAGCTGAGCT CGCTCGAGAC
      lyArgLeuIl eLysHisMet ThrMetAlaS erArgLeuGl uArgAlaLeu

         XmaI
         SmaIBamHI
801   CAGCCCGGGA TCCGGTAACT AACTAAGATC CGGTAAGATC CGGCTGCTAA
      GTCGGGCCCT AGGCCATTGA TTGATTCTAG GCCATTCTAG GCCGACGATT
      GlnProGlyIle Arg*** *  **  ***
```

*Figure 4. pIVEX2.4d vector map*(131).

*Table 2. Primers for pIVEX2.3d and 2.4d.*

| **For the plasmid vector pIVEX2.3d:** | **length** | **GC content** |
|---|---|---|
| **Forward primer: 5' GGGGGTTCTCATCATCATCAT 3'** | **21** | **47** |
| **Reverse primer: 5' GGTATATCTCCTTCTTAAAGTTAAAC 3'** | **26** | **30.7** |

| **For the plasmid vector pIVEX2.4d** | **length** | **GC content** |
|---|---|---|
| **Forward primer: 5' ACCATGGCAAAGTCGACTCGAGCG 3'** | **24** | **58** |
| **Reverse primer: 5' GATGCCGCTGCTATGATGATG 3'** | **21** | **52** |

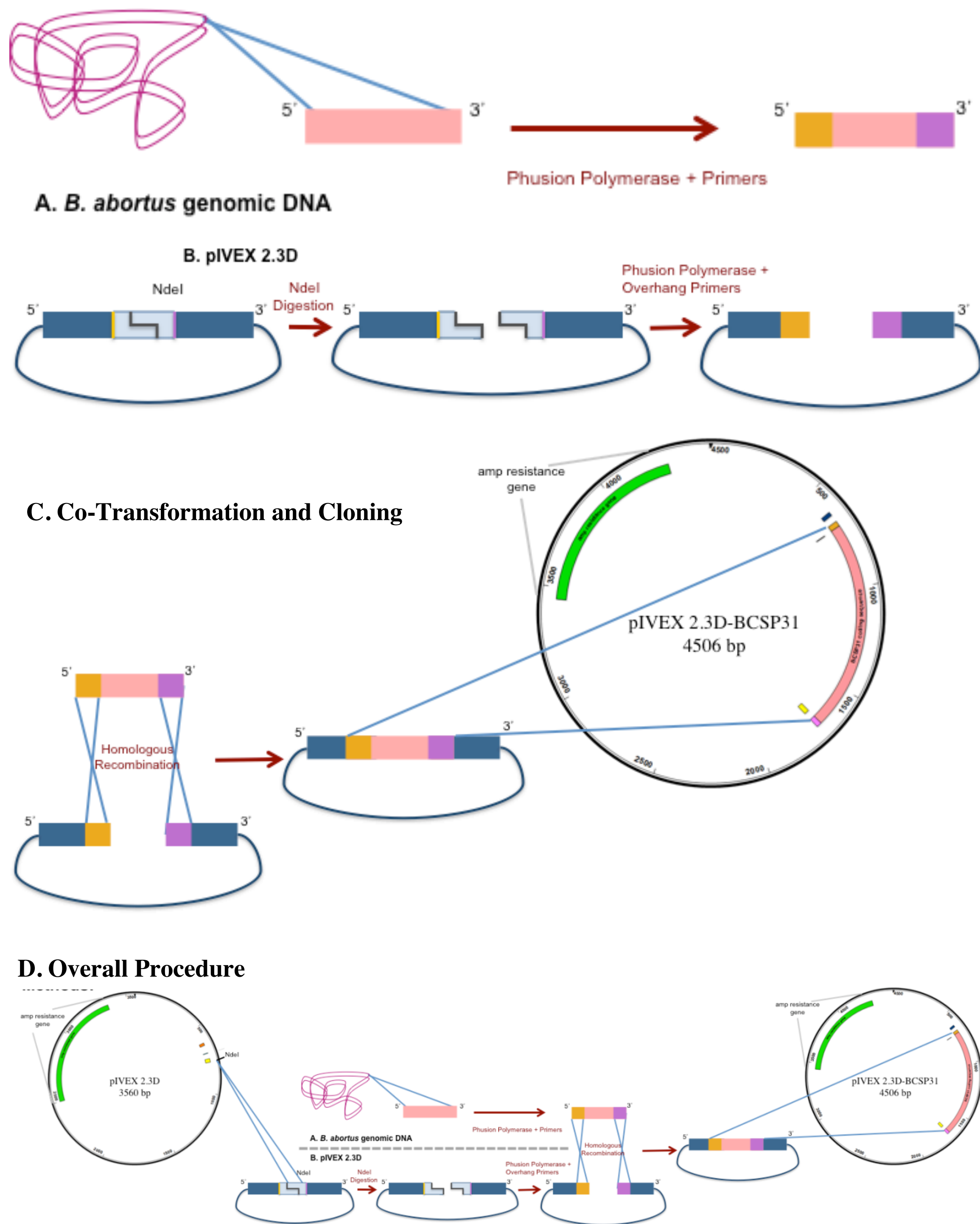



*Figure 5. In vivo cloning of a target gene into the expression vector pIVEX2.3d through homologous recombination. (A) A B. abortus target gene is synthesized by PCR with gene-specific primers, each containing a 25 or 26 nt stretch of sequence derived from the plasmid at the 5' and 3' end. (B) The plasmid vector is linearized with NdeI and amplified by PCR. (C) Transformation of E. coli DH5a with both PCR products results in cloning of the gene into the plasmid vector through homologous recombination in vivo. (D) Demonstrates the entirety of the process and how the three stages are linked.*

*Table 3. Primers designed for the BCSP31 protein, for use with pIVEX2.3d.*

| **For the BCSP31 protein into pIVEX2.3d:** | **Length** | **GC content** |
|---|---|---|
| **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAAATTCGGAAGCAAAAT 3'** | **46** | **30.5** |
| **R: 5' GATGATGATGATGATGAGAACCCCC TTTCAGCACGCCCGCTTCCT 3'** | **45** | **53.4** |

*Table 4. Primers designed for candidates for use with pIVEX2.3d.*

| Plasmid Constructs | Candidates | Primer Name | Primers used (5' to 3') |
|---|---|---|---|
| pIVEX2.3d | ALDH | LIN1432 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAACACCGCTGTTAGAAA 3'** |
| | | LIN1433 | **R: 5' GATGATGATGATGATGAGAACCCCC GTCGATATCGAAGGAAACAC 3'** |
| pIVEX2.3d | BCSP31 | LIN1434 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAAATTCGGAAGCAAAAT 3'** |
| | | LIN1435 | **R: 5' GATGATGATGATGATGAGAACCCCC TTTCAGCACGCCCGCTTCCT 3'** |
| pIVEX2.3d | CU-ZN SOD | LIN1436 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAAGTCCTTATTTATTGC 3'** |
| | | LIN1437 | **R: 5' GATGATGATGATGATGAGAACCCCC TTCGATCACGCCGCAGGCAA 3'** |
| pIVEX2.3d | VIRB12 | LIN1438 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCGCACATTGGTTATGGT 3'** |
| | | LIN1439 | **R: 5' GATGATGATGATGATGAGAACCCCC GATATCCACGCGCCTGTTCA 3'** |
| pIVEX2.3d | WP_002963597.1 | LIN1480 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAAAATGTGGACCCTTGC 3'** |
| | | LIN1481 | **R: 5' GATGATGATGATGATGAGAACCCCC CTGTTCTACGCAGCTTATAG 3'** |
| pIVEX2.3d | WP_002963836.1 | LIN1482 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCAATTGAACGTACTTT 3'** |
| | | LIN1483 | **R: 5' GATGATGATGATGATGAGAACCCCC GCCAACGATTTCGGTGCCCG 3'** |
| pIVEX2.3d | WP_002963844.1 | LIN1484 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCGCACTCTTAAGTCTCT 3'** |
| | | LIN1485 | **R: 5' GATGATGATGATGATGAGAACCCCC GAACTTGTAGCCGATGCCGA 3'** |
| pIVEX2.3d | WP_002963844.1 | LIN1486 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAAACGGAATTTCCAGAC 3'** |
| | | LIN1487 | **R: 5' GATGATGATGATGATGAGAACCCCC GGGCTCACTTCTCACACGCC 3'** |
| pIVEX2.3d | WP_002964008.1 | LIN1488 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGACGGACAGCAGTGCAAA 3'** |
| | | LIN1489 | **R: 5' GATGATGATGATGATGAGAACCCCC CTGCGTCACGAAACAGCTTC 3'** |
| pIVEX2.3d | WP_002964019.1 | LIN1492 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGACGAGTTGAACATGCG 3'** |
| | | LIN1493 | **R: 5' GATGATGATGATGATGAGAACCCCC TGATTCCAGATATTTGGTCG 3'** |

| | | | |
|---|---|---|---|
| pIVEX2.3d | WP_002964282.1 | LIN1494 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACGGCAAGTTCTAAATT 3’ |
| | | LIN1495 | R: 5’ GATGATGATGATGATGAGAACCCCC GAACTTTGTCGATACACCGA 3’ |
| pIVEX2.3d | WP_002964402.1 | LIN1496 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGTTCAAGCGTTCTATCAC 3’ |
| | | LIN1497 | R: 5’ GATGATGATGATGATGAGAACCCCC GAATTTGTAGTTCAGGCCGG 3’ |
| pIVEX2.3d | WP_002964530.1 | LIN1498 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACGAGTTTCAAATTCAC 3’ |
| | | LIN1499 | R: 5’ GATGATGATGATGATGAGAACCCCC TGAGCTGCCGCCGGTGATGA 3’ |
| pIVEX2.3d | WP_002964637.1 | LIN1500 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAAGCCGAACATGAACAA 3’ |
| | | LIN1501 | R: 5’ GATGATGATGATGATGAGAACCCCC TGCGCGATTTGCCGCAAGCC 3’ |
| pIVEX2.3d | WP_002964666.1 | LIN1502 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAACCGCTTCACCAAGAG 3’ |
| | | LIN1503 | R: 5’ GATGATGATGATGATGAGAACCCCC GAAACGGTAGGTAATACCGG 3’ |
| pIVEX2.3d | WP_002964719.1 | LIN1504 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGTTTAGCTTAAAAGGGAC 3’ |
| | | LIN1505 | R: 5’ GATGATGATGATGATGAGAACCCCC GAACTTGTAGTTCAGACCGA 3’ |
| pIVEX2.3d | WP_002964782.1 | LIN1506 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAGGAAACCAATGAGAAA 3’ |
| | | LIN1507 | R: 5’ GATGATGATGATGATGAGAACCCCC GCACTTGGCGCGACTGCGCT 3’ |
| pIVEX2.3d | WP_002964998.1 | LIN1508 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGGAATTTCAAAAGCAAG 3’ |
| | | LIN1509 | R: 5’ GATGATGATGATGATGAGAACCCCC GCGCGACAGCGTCACGGGCC 3’ |
| pIVEX2.3d | WP_002965367.1 | LIN1510 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACGTTCAAAAATCTACT 3’ |
| | | LIN1511 | R: 5’ GATGATGATGATGATGAGAACCCCC GAACTTATAGGCAACGCCGA 3’ |
| pIVEX2.3d | WP_002965368.1 | LIN1512 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAAGCTCAAGGCTCTTCT 3’ |
| | | LIN1513 | R: 5’ GATGATGATGATGATGAGAACCCCC GAACTTGTAAGCGACACCGA 3’ |
| pIVEX2.3d | WP_002969994.1 | LIN1514 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGTTCACCGCAATCGAGCA 3’ |
| | | LIN1515 | R: 5’ GATGATGATGATGATGAGAACCCCC GAATTTCACCTTGGCAGTGA 3’ |
| pIVEX2.3d | WP_002961512.1 | LIN1516 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAACATCAAGAGCCTTCT 3’ |
| | | LIN1517 | R: 5’ GATGATGATGATGATGAGAACCCCC GAACGAACGCTGGAAGCGAA 3’ |
| pIVEX2.3d | WP_002966502.1 | LIN1518 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAAACGCTTCCGCATCGT 3’ |
| | | LIN1519 | R: 5’ GATGATGATGATGATGAGAACCCCC GCCGGCGTTGCGGCGGGTGA 3’ |
| pIVEX2.3d | WP_002966591.1 | LIN1520 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAAGTTCACGAGAACGCT 3’ |
| | | LIN1521 | R: 5’ GATGATGATGATGATGAGAACCCCC GAACTTGAAGGCCGTCTGGA 3’ |
| pIVEX2.3d | WP_002966636.1 | LIN1522 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGCTAGCATTCTTACAAA 3’ |
| | | LIN1523 | R: 5’ GATGATGATGATGATGAGAACCCCC GCCGCGGAACAGCGACAGGA 3’ |

| pIVEX2.3d | WP_002966649.1 | LIN1524 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACGACGACATCTCCGGT 3’ |
|---|---|---|---|
| | | LIN1525 | R: 5’ GATGATGATGATGATGAGAACCCCC GCTGATCCTGACACCTTCGC 3’ |
| pIVEX2.3d | WP_002971189.1 | LIN1526 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAGAATAAAAGCTGTTAT 3’ |
| | | LIN1527 | R: 5’ GATGATGATGATGATGAGAACCCCC GAACTTGAAGTCGGTCTTGG 3’ |
| pIVEX2.3d | WP_002964049.1 | LIN1587 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAAAAGGATCGAGCGCCT 3’ |
| | | LIN1587 | R: 5’ GATGATGATGATGATGAGAACCCCC TTGGAACTCGAAGATGGAAC 3’ |
| pIVEX2.3d | WP_002964462.1 | LIN1588 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGCATATTCTGAAATTCTG 3’ |
| | | LIN1589 | R: 5’ GATGATGATGATGATGAGAACCCCC GAAATTACGGGTCAGCCCGA 3’ |
| pIVEX2.3d | WP_002965076.1 | LIN1590 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGGGGGAGAGAACAAGGT 3’ |
| | | LIN1591 | R: 5’ GATGATGATGATGATGAGAACCCCC GCTGTTGGTTCCTGTCAGAA 3’ |
| pIVEX2.3d | WP_002965376.1 | LIN1592 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGAAACGATCTCAACCGC 3’ |
| | | LIN1593 | R: 5’ GATGATGATGATGATGAGAACCCCC GCTGCGCAGCCGTTCCGAAT 3’ |
| pIVEX2.3d | WP_002966947.1 | LIN1594 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGCGCCGTATCCAGTCGAT 3’ |
| | | LIN1595 | R: 5’ GATGATGATGATGATGAGAACCCCC CCGTCCGGCCCCGTTGAGAA 3’ |
| pIVEX2.3d | WP_0025197938.1 | LIN1596 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC TTGGAGGGAACCGTGAACAT 3’ |
| | | LIN1597 | R: 5’ GATGATGATGATGATGAGAACCCCC CTCGGATGATGTGTCTTCAT 3’ |
| pIVEX2.3d | WP_002965683.1 | LIN1598 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGCTGCAATTGGCGATGCG 3’ |
| | | LIN1599 | R: 5’ GATGATGATGATGATGAGAACCCCC ACCCCGCGTGCGGGCCAGAC 3’ |
| pIVEX2.3d | HISD | LIN1694 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACCGACATGCAGAAACC 3’ |
| | | LIN1695 | R: 5’ GATGATGATGATGATGAGAACCCCC CTTCAGAAATTCTGTGAGGG 3’ |
| pIVEX2.3d | WP_002964749.1 | LIN1696 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACGTTATCGACGCGTAT 3’ |
| | | LIN1697 | R: 5’ GATGATGATGATGATGAGAACCCCC GGCAGCAACTTGCGATGCTT 3’ |
| pIVEX2.3d | WP_002966387.1 | LIN1698 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACGTTATCGACGCGTAT 3’ |
| | | LIN1699 | R: 5’ GATGATGATGATGATGAGAACCCCC GAAGTCCATGCCGCCCATGC 3’ |
| pIVEX2.3d | WP_002966987.1 | LIN1700 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGCGAAATCCGGCACCCC 3’ |
| | | LIN1701 | R: 5’ GATGATGATGATGATGAGAACCCCC CTGACCGGAAGAGGCCGGAG 3’ |
| pIVEX2.3d | WP_002966556.1 | LIN1702 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGTCCTTAAAAGTTTTCCT 3’ |
| | | LIN1703 | R: 5’ GATGATGATGATGATGAGAACCCCC GCCCGGACGCTTCATGGATG 3’ |
| pIVEX2.3d | WP_0029646401.1 | LIN1704 | F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGCATTTCTCGCCGACGC 3’ |
| | | LIN1705 | R: 5’ GATGATGATGATGATGAGAACCCCC GCGCAGGCTGGCGCAGAAAC 3’ |

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| pIVEX2.3d | WP_002963897.1 | LIN1706 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCAGATTGACTTCGGCGG 3'** |
| | | LIN1707 | **R: 5' GATGATGATGATGATGAGAACCCCC CAATGTCAGTTCCCTGTCAC 3'** |
| pIVEX2.3d | WP_002964288.1 | LIN1708 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAGCATTTCCGCATCTCT 3'** |
| | | LIN1709 | **R: 5' GATGATGATGATGATGAGAACCCCC GCCCTTGGCGGCTGCGGCCA 3'** |
| pIVEX2.3d | WP_002964995.1 | LIN1710 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCACGCAACAAGATTGC 3'** |
| | | LIN1711 | **R: 5' GATGATGATGATGATGAGAACCCCC TTTCAGCGACGGAGCAATAC 3'** |
| pIVEX2.3d | WP_002964841.1 | LIN1712 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCGACTTTTATCCTTGCT 3'** |
| | | LIN1713 | **R: 5' GATGATGATGATGATGAGAACCCCC TCTGCTGGTGGCTGCCAGCC 3'** |
| pIVEX2.3d | WP_002964172.1 | LIN1716 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTTCAATTCGGTACTCGA 3'** |
| | | LIN1717 | **R: 5' GATGATGATGATGATGAGAACCCCC CCCCTCGAACGGTATGTCAT 3'** |
| pIVEX2.3d | WP_002965705.1 | LIN1718 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTTGCGTCTGGTACAGTT 3'** |
| | | LIN1719 | **R: 5' GATGATGATGATGATGAGAACCCCC CAATGCGCGCACGGTGATTT 3'** |
| pIVEX2.3d | WP_002964289.1 | LIN1720 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCATTGCCTGATTTCAG 3'** |
| | | LIN1721 | **R: 5' GATGATGATGATGATGAGAACCCCC GGCGCCTTCGGCCGGAGCCT 3'** |
| pIVEX2.3d | WP_002965601.1 | LIN1722 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAGCGAGGAAGTGCAAGG 3'** |
| | | LIN1723 | **R: 5' GATGATGATGATGATGAGAACCCCC CGTATTGAGCCCGAGCACGT 3'** |
| pIVEX2.3d | WP_002966987.1 | LIN1724 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCGAAATCCGGCACCCC 3'** |
| | | LIN1725 | **R: 5' GATGATGATGATGATGAGAACCCCC CTGACCGGAAGAGGCCGGAG 3'** |
| pIVEX2.3d | WP_002966988.1 | LIN1726 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCGCGTTCTTGGAATAGA 3'** |
| | | LIN1727 | **R: 5' GATGATGATGATGATGAGAACCCCC TGCCTTTGCTCCCCGCCGTC 3'** |
| pIVEX2.3d | WP_002967016.1 | LIN1728 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCTGTATCGGCAGTAAT 3'** |
| | | LIN1729 | **R: 5' GATGATGATGATGATGAGAACCCCC TATTACCCTTGTAGAAAGAA 3'** |
| pIVEX2.3d | WP_002969883.1 | LIN1730 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAACATCAAGAGCCTTCT 3'** |
| | | LIN1731 | **R: 5' GATGATGATGATGATGAGAACCCCC GAACGAGCGCTGGAAGCGAA 3'** |
| pIVEX2.3d | WP_002967131.1 | LIN1732 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTCACTTAGTTCTGCTCT 3'** |
| | | LIN1733 | **R: 5' GATGATGATGATGATGAGAACCCCC CACCGCGTTAAGAAGATCAT 3'** |
| pIVEX2.3d | WP_002973384.1 | LIN1734 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCCGATGGGACCGAAAGA 3'** |
| | | LIN1735 | **R: 5' GATGATGATGATGATGAGAACCCCC ATGACTTGAGGAACCCGGCG 3'** |
| pIVEX2.3d | WP_002963464.1 | LIN1736 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAACTGGACAGACGAGCG 3'** |
| | | LIN1737 | **R: 5' GATGATGATGATGATGAGAACCCCC GCGCGCCCGGCGGCGTTCGG 3'** |

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|---|---|---|---|
| pIVEX2.3d | WP_002963473.1 | LIN1738 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCAGCAAAAACATGGAAT 3'** |
| | | LIN1739 | **R: 5' GATGATGATGATGATGAGAACCCCC CAGGCCGAGCATCATCGCCC 3'** |
| pIVEX2.3d | WP_002963488.1 | LIN1740 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGAAGGTCAAGTCATATT 3'** |
| | | LIN1741 | **R: 5' GATGATGATGATGATGAGAACCCCC GACGTCACCGGGTTTCTTGA 3'** |
| pIVEX2.3d | WP_002963504.1 | LIN1742 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAAAAATTATCGTGCAAT 3'** |
| | | LIN1743 | **R: 5' GATGATGATGATGATGAGAACCCCC CTTGGTCAATGCCTGAATGC 3'** |
| pIVEX2.3d | WP_002963512.1 | LIN1744 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGGTCAGGAGCAGAACGG 3'** |
| | | LIN1745 | **R: 5' GATGATGATGATGATGAGAACCCCC GCTCCCCCAGCATGTCGAAC 3'** |
| pIVEX2.3d | WP_002963525.1 | LIN1746 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGATTACAATTCCCTGCG 3'** |
| | | LIN1747 | **R: 5' GATGATGATGATGATGAGAACCCCC GTCATTTTTGTCGTCCTTGA 3'** |
| pIVEX2.3d | WP_002963529.1 | LIN1748 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTTTATAAAGGTCGAGAA 3'** |
| | | LIN1749 | **R: 5' GATGATGATGATGATGAGAACCCCC TAGGGCTCGAAGCAGTTTCG 3'** |
| pIVEX2.3d | WP_002963545.1 | LIN1750 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTTCGTGTCCACGGCGTT 3'** |
| | | LIN1751 | **R: 5' GATGATGATGATGATGAGAACCCCC GGCGTTCGAGGCCCGGATGG 3'** |
| pIVEX2.3d | WP_0029635456.1 | LIN1752 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGACGCAACATTCTGGGC 3'** |
| | | LIN1753 | **R: 5' GATGATGATGATGATGAGAACCCCC GTTCAGGTGCGACTTCACCT 3'** |
| pIVEX2.3d | WP_002963553.1 | LIN1754 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGACAGCATTCCAGAACGC 3'** |
| | | LIN1755 | **R: 5' GATGATGATGATGATGAGAACCCCC GGCTCTGGAAGGAAAGCTGT 3'** |
| pIVEX2.3d | WP_002963554.1 | LIN1756 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCCCCAGCTTATATTCCT 3'** |
| | | LIN1757 | **R: 5' GATGATGATGATGATGAGAACCCCC ATCCTTGCGGACGTAATATT 3'** |
| pIVEX2.3d | WP_002963558.1 | LIN1758 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGATCGAGCTTATCCGCAC 3'** |
| | | LIN1759 | **R: 5' GATGATGATGATGATGAGAACCCCC TTTATTATCGCGCAATTCGT 3'** |
| pIVEX2.3d | WP_002963567.1 | LIN1760 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGATGAGCAAGGGACTGAA 3'** |
| | | LIN1761 | **R: 5' GATGATGATGATGATGAGAACCCCC ATGACGAGCAATGATTTCCT 3'** |
| pIVEX2.3d | WP_002963581.1 | LIN1762 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGGCACTCTCGATTTCCG 3'** |
| | | LIN1763 | **R: 5' GATGATGATGATGATGAGAACCCCC TTTTTGCTGCTTGGTAGAGG 3'** |
| pIVEX2.3d | WP_002963582.1 | LIN1764 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTATCAGGATAAGGAAAG 3'** |
| | | LIN1765 | **R: 5' GATGATGATGATGATGAGAACCCCC TTTTCTTTTCGATGCCACTT 3'** |
| pIVEX2.3d | WP_002963584.1 | LIN1766 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCATTATCTGATCGGATT 3'** |
| | | LIN1767 | **R: 5' GATGATGATGATGATGAGAACCCCC CGGGGCTTCCTGAATATCGG 3'** |

| pIVEX2.3d | WP_002963592.1 | LIN1768 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC GTGTTTCTGGAACGGTGGGA 3’** |
|---|---|---|---|
| | | LIN1769 | **R: 5’ GATGATGATGATGATGAGAACCCCC TTCTGGCAAGGGAATGAAAT 3’** |
| pIVEX2.3d | WP_002963624.1 | LIN1770 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGTTTTCCGTATGTTTGC 3’** |
| | | LIN1771 | **R: 5’ GATGATGATGATGATGAGAACCCCC GGGGAGGGCGTCGAAGCCTT 3’** |
| pIVEX2.3d | WP_002963635.1 | LIN1772 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC GTGAAGCGCATCGAGGCCTA 3’** |
| | | LIN1773 | **R: 5’ GATGATGATGATGATGAGAACCCCC TTTCACCTTCAACGTGCGGT 3’** |
| pIVEX2.3d | WP_002966739.1 | LIN1876 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGTATTGCCCCATACGCT 3’** |
| | | LIN1877 | **R: 5’ GATGATGATGATGATGAGAACCCCC GAAAGTCTGCGAACCGTTGC 3’** |
| pIVEX2.3d | WP_002969501.1 | LIN1878 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC GTGATCGTGGATTCCAATTC 3’** |
| | | LIN1879 | **R: 5’ GATGATGATGATGATGAGAACCCCC CCACCTCACCCTGAAACCAA 3’** |
| pIVEX2.3d | WP_002969598.1 | LIN1880 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC TTGGGCGGCTATTCCGATGTGA 3’** |
| | | LIN1881 | **R: 5’ GATGATGATGATGATGAGAACCCCC AAAGCCCCCGTAGAGGCTGA 3’** |
| pIVEX2.3d | WP_002971772.1 | LIN1882 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGCTGTTGCTGACGGCGCA 3’** |
| | | LIN1883 | **R: 5’ GATGATGATGATGATGAGAACCCCC AAACGCCTGTCCTATGCCTA 3’** |
| pIVEX2.3d | WP_040120204.1 | LIN1884 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGCCTGGTCTTTTTAGCTC 3’** |
| | | LIN1885 | **R: 5’ GATGATGATGATGATGAGAACCCCC ATTGAAGGTATAGCTGAAAC 3’** |
| pIVEX2.3d | WP_002967178.1 | LIN1886 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGTTTATTGCTTGTGCTCC 3’** |
| | | LIN1887 | **R: 5’ GATGATGATGATGATGAGAACCCCC TTTCTGGAATTTCATGCCGA 3’** |
| pIVEX2.3d | WP_002968802.1 | LIN1888 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAGCCTCTACGGTATGAT 3’** |
| | | LIN1889 | **R: 5’ GATGATGATGATGATGAGAACCCCC TCTCTTCAGATTAACCAGCA 3’** |
| pIVEX2.3d | WP_002965266.1 | LIN1890 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGCCGGTTCTGAAATCTGA 3’** |
| | | LIN1891 | **R: 5’ GATGATGATGATGATGAGAACCCCC CATTCTGAAGATGCCAAAAC 3’** |
| pIVEX2.3d | WP_002963577.1 | LIN1892 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACCGCACAGTCTGGTTT 3’** |
| | | LIN1893 | **R: 5’ GATGATGATGATGATGAGAACCCCC GTCTTTCACGACCGAGATAT 3’** |
| pIVEX2.3d | WP_002967114.1 | LIN1894 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGAGGGAGCCGACCTTGGA 3’** |
| | | LIN1895 | **R: 5’ GATGATGATGATGATGAGAACCCCC TCCTGTCACGCCTACATCCG 3’** |
| pIVEX2.3d | WP_002964293.1 | LIN1896 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGGCCTCCTCCGATAAATC 3’** |
| | | LIN1897 | **R: 5’ GATGATGATGATGATGAGAACCCCC AGCAATGAGGGCCCGCGGAT 3’** |
| pIVEX2.3d | WP_002970984.1 | LIN1898 | **F: 5’ GTTTAACTTTAAGAAGGAGATATACC ATGACAGATGAAACTGTAAC 3’** |
| | | LIN1899 | **R: 5’ GATGATGATGATGATGAGAACCCCC AAGCCTCCGCGCGCCGCGCC 3’** |

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| pIVEX2.3d | WP_002965534.1 | LIN1900 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGTCACAACGCTCAGACA 3'** |
| | | LIN1901 | **R: 5' GATGATGATGATGATGAGAACCCCC TAGGTTCAGACGAATGGCGA 3'** |
| pIVEX2.3d | WP_002966968.1 | LIN1902 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCAAAAGCAGCGACCCC 3'** |
| | | LIN1903 | **R: 5' GATGATGATGATGATGAGAACCCCC GGCAGCTTCAGCCGCCAGCT 3'** |
| pIVEX2.3d | WP_002971551.1 | LIN1904 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCCTGTAGCGCTTAATCG 3'** |
| | | LIN1905 | **R: 5' GATGATGATGATGATGAGAACCCCC AAGAGCGCTGTCGATGAATC 3'** |
| pIVEX2.3d | WP_002963760.1 | LIN1906 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCCTGTAGCGCTTAATCG 3'** |
| | | LIN1907 | **R: 5' GATGATGATGATGATGAGAACCCCC AAGAGCGCTGTCGATGAATC 3'** |
| pIVEX2.3d | WP_002968051.1 | LIN1908 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCAGAACAGAAATCGAG 3'** |
| | | LIN1909 | **R: 5' GATGATGATGATGATGAGAACCCCC TTGCGCGCTGCCTTCCTTGG 3'** |
| pIVEX2.3d | WP_0029667970.1 | LIN1910 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAAGTTTTTCGTGGACAC 3'** |
| | | LIN1911 | **R: 5' GATGATGATGATGATGAGAACCCCC GGCGATCTTCTGGCCGGTCT 3'** |
| pIVEX2.3d | WP_002965390.1 | LIN1912 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAGCACGTTCAAACCGCT 3'** |
| | | LIN1913 | **R: 5' GATGATGATGATGATGAGAACCCCC GTTCTGCAACCAGCCGACAA 3'** |
| pIVEX2.3d | WP_002963466.1 | LIN1914 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCAAACGATAACGGTAT 3'** |
| | | LIN1915 | **R: 5' GATGATGATGATGATGAGAACCCCC TGCTCCCAAACCGCGGTCTT 3'** |
| pIVEX2.3d | WP_002963831.1 | LIN1916 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTCTAAACGTCCTTCGAT 3'** |
| | | LIN1917 | **R: 5' GATGATGATGATGATGAGAACCCCC GCTTTCAAATTGATCGCGGA 3'** |
| pIVEX2.3d | WP_002969557.1 | LIN1918 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGTAACCTATATCGAAGC 3'** |
| | | LIN1919 | **R: 5' GATGATGATGATGATGAGAACCCCC CCGCATGGAAGGCGGGCCAA 3'** |
| pIVEX2.3d | WP_002963876.1 | LIN1920 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTACAATTTATTTGTTTC 3'** |
| | | LIN1921 | **R: 5' GATGATGATGATGATGAGAACCCCC GGTGATGAGGGCGACGCGCT 3'** |
| pIVEX2.3d | WP_002965779.1 | LIN1922 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCAGATGCAGACGATTC 3'** |
| | | LIN1923 | **R: 5' GATGATGATGATGATGAGAACCCCC TTTTCCATTCGGCTGGCCGA 3'** |
| pIVEX2.3d | WP_002965780.1 | LIN1924 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGGCCCTGACGCTTTCGCT 3'** |
| | | LIN1925 | **R: 5' GATGATGATGATGATGAGAACCCCC TATCTTCAAGTCCTTAGCGG 3'** |
| pIVEX2.3d | WP_002965788.1 | LIN1926 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTCAGTCACGAGCACCAA 3'** |
| | | LIN1927 | **R: 5' GATGATGATGATGATGAGAACCCCC TCGCCGGGAATATTGAATGC 3'** |
| pIVEX2.3d | WP_002968536.1 | LIN1928 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAGGCATATTATTTTCGG 3'** |
| | | LIN1929 | **R: 5' GATGATGATGATGATGAGAACCCCC GGCCGCTTTTGACGGGCTCA 3'** |

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| pIVEX2.3d | WP_002965581.1 | LIN1930 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGCAGACCCGATATCCAGA 3'** |
| | | LIN1931 | **R: 5' GATGATGATGATGATGAGAACCCCC GGCCTGCAGAAAGGAAACGA 3'** |
| pIVEX2.3d | WP_002967303.1 | LIN1932 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGTATCGCAAATTTCTACT 3'** |
| | | LIN1933 | **R: 5' GATGATGATGATGATGAGAACCCCC TTTTTTAAGGGAAAGCCAAC 3'** |
| pIVEX2.3d | WP_002966589.1 | LIN1934 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGATAGGAACTCGTTTGCC 3'** |
| | | LIN1935 | **R: 5' GATGATGATGATGATGAGAACCCCC ACGCGCATTCCAGGCAGGCA 3'** |
| pIVEX2.3d | WP_002972147.1 | LIN1936 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGATACTGCCCGAGCGCCT 3'** |
| | | LIN1937 | **R: 5' GATGATGATGATGATGAGAACCCCC GACAAGCGCATGTAGAGAAG 3'** |
| pIVEX2.3d | WP_003966033.1 | LIN1938 | **F: 5' GTTTAACTTTAAGAAGGAGATATACC ATGAGCGCGATCCTGTTTGA 3'** |
| | | LIN1939 | **R: 5' GATGATGATGATGATGAGAACCCCC AGCCGAATAAACGGTCTTGC 3'** |

*Table 5. Primers designed for candidates for use with pIVEX2.4d.*

| PLASMID CONSTRUCTS | CANDIDATES | PRIMER NAME | PRIMERS USED (5' TO 3') |
|---|---|---|---|
| pIVEX2.4d | WP_002963597.1 | LIN2014 | **F: 5' CATCATCATAGCAGCGGCATC ATGAACACCGCTGTTAGAAA 3'** |
| | | LIN2015 | **R': 5' CGCTCGAGTCGACTTGCCATGGT TCACTGTTCTACGCAGCTTATAG 3'** |
| pIVEX2.4d | WP_002963836.1 | LIN2016 | **F: 5' CATCATCATAGCAGCGGCATC ATGGCAATTGAACGTACTTT 3'** |
| | | LIN2017 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGCCAACGATTTCGGTGCCCG 3'** |
| pIVEX2.4d | WP_002963844.1 | LIN2018 | **F: 5' CATCATCATAGCAGCGGCATC ATGCGCACTCTTAAGTCTCT 3'** |
| | | LIN2019 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTAGAACTTGTAGCCGATGCCGA 3'** |
| pIVEX2.4d | WP_002963986.1 | LIN2020 | **F: 5' CATCATCATAGCAGCGGCATC ATGAAACGGAATTTCCAGAC 3'** |
| | | LIN2021 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGGGCTCACTTCTCACACGCC 3'** |
| pIVEX2.4d | WP_002964402.1 | LIN2022 | **F: 5' CATCATCATAGCAGCGGCATC ATGTTCAAGCGTTCTATCAC 3'** |
| | | LIN2023 | **R':5' CGCTCGAGTCGACTTGCCATGGT CTAGAATTTGTAGTTCAGGCCGG 3'** |
| pIVEX2.4d | WP_002964530.1 | LIN2024 | **F: 5' CATCATCATAGCAGCGGCATC ATGACGAGTTTCAAATTCAC 3'** |
| | | LIN2025 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTATGAGCTGCCGCCGGTGATGA 3'** |
| pIVEX2.4d | WP_002964637.1 | LIN2026 | **F: 5' CATCATCATAGCAGCGGCATC ATGAAGCCGAACATGAACAA 3'** |
| | | LIN2027 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCATGCGCGATTTGCCGCAAGCC 3'** |
| pIVEX2.4d | WP_002964666.1 | LIN2028 | **F: 5' CATCATCATAGCAGCGGCATC ATGAACCGCTTCACCAAGAG 3'** |

|  |  | LIN2029 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGAAACGGTAGGTAATACCGG 3'** |
|---|---|---|---|
| pIVEX2.4d | WP_002964719.1 | LIN2030 | **F: 5' CATCATCATAGCAGCGGCATC ATGTTTAGCTTAAAAGGGAC 3'** |
|  |  | LIN2031 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTAGAACTTGTAGTTCAGACCGA 3'** |
| pIVEX2.4d | WP_002964998.1 | LIN2032 | **F: 5' CATCATCATAGCAGCGGCATC ATGGGAATTTCAAAAGCAAG 3'** |
|  |  | LIN2033 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGCGCGACAGCGTCACGGCCT 3'** |
| pIVEX2.4d | WP_002965367.1 | LIN2034 | **F: 5' CATCATCATAGCAGCGGCATC ATGACGTTCAAAAATCTACT 3'** |
|  |  | LIN2035 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGAACTTATAGGCAACGCCGA 3'** |
| pIVEX2.4d | WP_002965368.1 | LIN2036 | **F: 5' CATCATCATAGCAGCGGCATC ATGAAGCTCAAGGCTCTTCT 3'** |
|  |  | LIN2037 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGAACTTGTAAGCGACACCGA 3'** |
| pIVEX2.4d | WP_002969994.1 | LIN2038 | **F: 5' CATCATCATAGCAGCGGCATC ATGTTCACCGCAATCGAGCA 3'** |
|  |  | LIN2039 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTAGAATTTCACCTTGGCAGTGA 3'** |
| pIVEX2.4d | WP_002966502.1 | LIN2040 | **F: 5' CATCATCATAGCAGCGGCATC ATGAAACGCTTCCGCATCGT 3'** |
|  |  | LIN2041 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGCCGGCGTTGCGGCGGGTGA 3'** |
| pIVEX2.4d | WP_002966636.1 | LIN2042 | **F: 5' CATCATCATAGCAGCGGCATC ATGGCTAGCATTCTTACAAA 3'** |
|  |  | LIN2043 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTAGCCGCGGAACAGCGACAGGA 3'** |
| pIVEX2.4d | WP_002966649.1 | LIN2044 | **F: 5' CATCATCATAGCAGCGGCATC ATGACGACGACATCTCCGGT 3'** |
|  |  | LIN2045 | **R':5' CGCTCGAGTCGACTTGCCATGGT CTAGCTGATCCTGACACCTTCGC 3'** |
| pIVEX2.4d | WP_002965076.1 | LIN2046 | **F: 5' CATCATCATAGCAGCGGCATC ATGGGGGGAGAGAACAAGGT 3'** |
|  |  | LIN2047 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTAGCTGTTGGTTCCTGTCAGAA 3'** |
| pIVEX2.4d | WP_002963384.1 | LIN2048 | **F: 5' CATCATCATAGCAGCGGCATC ATGCCGATGGGACCGAAAGA 3'** |
|  |  | LIN2049 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAATGACTTGAGGAACCCGGCG 3'** |
| pIVEX2.4d | WP_002963512.1 | LIN2050 | **F: 5' CATCATCATAGCAGCGGCATC ATGGGTCAGGAGCAGAACGG 3'** |
|  |  | LIN2051 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGCTCCCCCAGCATGTCGAAC 3'** |
| pIVEX2.4d | WP_002963525.1 | LIN2052 | **F: 5' CATCATCATAGCAGCGGCATC ATGGATTACAATTCCCTGCG 3'** |
|  |  | LIN2053 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGTCATTTTTGTCGTCCTTGA 3'** |
| pIVEX2.4d | WP_002963529.1 | LIN2054 | **F: 5' CATCATCATAGCAGCGGCATC ATGTTTATAAAGGTCGAGAA 3'** |
|  |  | LIN2055 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCATAGGGCTCGAAGCAGTTTCG 3'** |
| pIVEX2.4d | WP_002963558.1 | LIN2056 | **F: 5' CATCATCATAGCAGCGGCATC ATGATCGAGCTTATCCGCAC 3'** |
|  |  | LIN2057 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCATTTATTATCGCGCAATTCGT 3'** |
| pIVEX2.4d | WP_002963581.1 | LIN2058 | **F: 5' CATCATCATAGCAGCGGCATC ATGGGCACTCTCGATTTCCG 3'** |

| | | | |
|---|---|---|---|
| | | LIN2059 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTATTTTTGCTGCTTGGTAGAGG 3'** |
| pIVEX2.4d | WP_002963582.1 | LIN2060 | **F: 5' CATCATCATAGCAGCGGCATC ATGTATCAGGATAAGGAAAG 3'** |
| | | LIN2061 | **R':5' CGCTCGAGTCGACTTGCCATGGT CTATTTTCTTTTCGATGCCACTT 3'** |
| pIVEX2.4d | WP_002963584.1 | LIN2062 | **F: 5' CATCATCATAGCAGCGGCATC ATGCATTATCTGATCGGATT 3'** |
| | | LIN2063 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTACGGGGCTTCCTGAATATCGG 3'** |
| pIVEX2.4d | WP_002963464.1 | LIN2064 | **F: 5' CATCATCATAGCAGCGGCATC ATGAACTGGACAGACGAGCG 3'** |
| | | LIN2065 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGCGCGCCCGGCGGCGTTCGG 3'** |
| pIVEX2.4d | WP_002963473.1 | LIN2066 | **F: 5' CATCATCATAGCAGCGGCATC ATGCAGCAAAAACATGGAAT 3'** |
| | | LIN2067 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTACAGGCCGAGCATCATCGCCC 3'** |
| pIVEX2.4d | WP_002963504.1 | LIN2068 | **F: 5' CATCATCATAGCAGCGGCATC ATGAAAAATTATCGTGCAAT 3'** |
| | | LIN2069 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTACTTGGTCAATGCCTGAATGC 3'** |
| pIVEX2.4d | WP_002963545.1 | LIN2070 | **F: 5' CATCATCATAGCAGCGGCATC ATGTTCGTGTCCACGGCGTT 3'** |
| | | LIN2071 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGGCGTTCGAGGCCCGGATCC 3'** |
| pIVEX2.4d | WP_002963546.1 | LIN2072 | **F: 5' CATCATCATAGCAGCGGCATC ATGGACGCAACATTCTGGGC 3'** |
| | | LIN2073 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGTTCAGGTGCGACTTCACCT 3'** |
| pIVEX2.4d | WP_002963553.1 | LIN2074 | **F: 5' CATCATCATAGCAGCGGCATC ATGACAGCATTCCAGAACGC 3'** |
| | | LIN2075 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGGCTCTGGAAGGAAAGCTGT 3'** |
| pIVEX2.4d | WP_002963592.1 | LIN2076 | **F: 5' CATCATCATAGCAGCGGCATC GTGTTTCTGGAACGGTGGGA 3'** |
| | | LIN2077 | **R':5' CGCTCGAGTCGACTTGCCATGGT CTATTCTGGCAAGGGAATGAAAT 3'** |
| pIVEX2.4d | WP_002963635.1 | LIN2078 | **F: 5' CATCATCATAGCAGCGGCATC GTGAAGCGCATCGAGGCCTA 3'** |
| | | LIN2079 | **R':5' CGCTCGAGTCGACTTGCCATGGT CTATTTCACCTTCAACGTGCGGT 3'** |
| pIVEX2.4d | WP_002963577.1 | LIN2417 | **F: 5' CATCATCATAGCAGCGGCATC ATGACCGCACAGTCTGGTTT 3'** |
| | | LIN2418 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGTCTTTCACGACCGAGATAT 3'** |
| pIVEX2.4d | WP_002967114.1 | LIN2419 | **F: 5' CATCATCATAGCAGCGGCATC ATGAGGGAGCCGACCTTGGA 3'** |
| | | LIN2420 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTATCCTGTCACGCCTACATCCG 3'** |
| pIVEX2.4d | WP_002964293.1 | LIN2421 | **F: 5' CATCATCATAGCAGCGGCATC ATGGCCTCCTCCGATAAATC 3'** |
| | | LIN2422 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAAGCAATGAGGGCCCGCGGAT 3'** |
| pIVEX2.4d | WP_002970984.1 | LIN2423 | **F: 5' CATCATCATAGCAGCGGCATC ATGACAGATGAAACTGTAAC 3'** |
| | | LIN2424 | **R':5' CGCTCGAGTCGACTTGCCATGGT UCAAAGCCTCCGCGCGCCGCGCC 3'** |
| pIVEX2.4d | WP_002971551.1 | LIN2425 | **F: 5' CATCATCATAGCAGCGGCATC ATGCCTGTAGCGCTTAATCG 3'** |

| | | LIN2426 | R':5' CGCTCGAGTCGACTTGCCATGGT TCAAAGAGCGCTGTCGATGAATC 3' |
|---|---|---|---|
| pIVEX2.4d | WP_002963876.1 | LIN2427 | F: 5' CATCATCATAGCAGCGGCATC ATGTACAATTTATTTGTTTC 3' |
| | | LIN2428 | R':5' CGCTCGAGTCGACTTGCCATGGT CTAGGTGATGAGGGCGACGCGCT 3' |
| pIVEX2.4d | WP_002965779.1 | LIN2429 | F: 5' CATCATCATAGCAGCGGCATC ATGGCAGATGCAGACGATTC 3' |
| | | LIN2430 | R':5' CGCTCGAGTCGACTTGCCATGGT TTATTTTCCATTCGGCTGGCCGA 3' |
| pIVEX2.4d | WP_002972147.1 | LIN2431 | F: 5' CATCATCATAGCAGCGGCATC ATGATACTGCCCGAGCGCCT 3' |
| | | LIN2432 | R':5' CGCTCGAGTCGACTTGCCATGGT TCAGACAAGCGCATGTAGAGAAG 3' |
| pIVEX2.4d | WP_002964782.1 | LIN2451 | F: 5' CATCATCATAGCAGCGGCATC AGGAAACCAATGAGAAAAGT 3' |
| | | LIN2452 | R':5' CGCTCGAGTCGACTTGCCATGGT TCAGCACTTGGCGCGACTGCGCT 3' |
| pIVEX2.4d | WP_002964462.1 | LIN2453 | F: 5' CATCATCATAGCAGCGGCATC CATATTCTGAAATTGTCAGC 3' |
| | | LIN2454 | R':5' CGCTCGAGTCGACTTGCCATGGT TCAGAAATTACGGGTCAGCCCGA 3' |
| pIVEX2.4d | WP_002966987.1 | LIN2455 | F: 5' CATCATCATAGCAGCGGCATC GCGAAATCCGGCACCCCGCG 3' |
| | | LIN2456 | R':5' CGCTCGAGTCGACTTGCCATGGT TTACTGACCGGAAGAGGCCGGAG 3' |
| pIVEX2.4d | WP_002967131.1 | LIN2457 | F: 5' CATCATCATAGCAGCGGCATC TCACTTAGTTCTGCTCTTCT 3' |
| | | LIN2458 | R':5' CGCTCGAGTCGACTTGCCATGGT TCACACCGCGTTAAGAAGATCAT 3' |
| pIVEX2.4d | WP_002969598.1 | LIN2459 | F: 5' CATCATCATAGCAGCGGCATC GGCGGCTATTCCGATGTGAA 3' |
| | | LIN2460 | R':5' CGCTCGAGTCGACTTGCCATGGT CTAAAAGCCCCCGTAGAGGCTGA 3' |
| pIVEX2.4d | WP_002971772.1 | LIN2461 | F: 5' CATCATCATAGCAGCGGCATC CTGTTGCTGACGGCGCATTT 3' |
| | | LIN2462 | R':5' CGCTCGAGTCGACTTGCCATGGT TCAAAACGCCTGTCCTATGCCTA 3' |
| pIVEX2.4d | WP_002967168.1 | LIN2463 | F: 5' CATCATCATAGCAGCGGCATC TTTATTGCTTGTGCTCCCAT 3' |
| | | LIN2464 | R':5' CGCTCGAGTCGACTTGCCATGGT TTATTTCTGGAATTTCATGCCGA 3' |
| pIVEX2.4d | WP_002968802.1 | LIN2465 | F: 5' CATCATCATAGCAGCGGCATC AGCCTCTACGGTATGATGCG 3' |
| | | LIN2466 | R':5' CGCTCGAGTCGACTTGCCATGGT TTATCTCTTCAGATTAACCAGCA 3' |
| pIVEX2.4d | WP_002965534.1 | LIN2467 | F: 5' CATCATCATAGCAGCGGCATC GTCACAACGCTCAGACAGAC 3' |
| | | LIN2468 | R':5' CGCTCGAGTCGACTTGCCATGGT TCATAGGTTCAGACGAATGGCGA 3' |
| pIVEX2.4d | WP_002963760.1 | LIN2469 | F: 5' CATCATCATAGCAGCGGCATC TCCAGAGCCAGGATTTCCA 3' |
| | | LIN2470 | R':5' CGCTCGAGTCGACTTGCCATGGT TCATTCCTGATTGATCGGCAGCG 3' |
| pIVEX2.4d | WP_002965390.1 | LIN2471 | F: 5' CATCATCATAGCAGCGGCATC AGCACGTTCAAACCGCTTGT 3' |
| | | LIN2472 | R':5' CGCTCGAGTCGACTTGCCATGGT TCAGTTCTGCAACCAGCCGACAA 3' |
| pIVEX2.4d | WP_002965788.1 | LIN2473 | F: 5' CATCATCATAGCAGCGGCATC TCAGTCACGAGCACCAACAA 3' |

| | | | |
|---|---|---|---|
| | | LIN2474 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCATCGCCGGGAATATTGAATGC 3'** |
| pIVEX2.4d | WP_002966033.1 | LIN2475 | **F: 5' CATCATCATAGCAGCGGCATC AGCGCGATCCTGTTTGAAAA 3'** |
| | | LIN2476 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAAGCCGAATAAACGGTCTTGC 3'** |
| pIVEX2.4d | WP_002964282.1 | LIN2477 | **F: 5' CATCATCATAGCAGCGGCATC ACGGCAAGTTCTAAATTCTT 3'** |
| | | LIN2478 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGAACTTTGTCGATACACCGA 3'** |
| pIVEX2.4d | WP_002965076.1 | LIN2479 | **F: 5' CATCATCATAGCAGCGGCATC GGGGGAGAGAACAAGGTGCC 3'** |
| | | LIN2480 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTAGCTGTTGGTTCCTGTCACTT 3'** |
| pIVEX2.4d | WP_002966739.1 | LIN2481 | **F: 5' CATCATCATAGCAGCGGCATC GTATTGCCCCATACGCTCTC 3'** |
| | | LIN2482 | **R':5' CGCTCGAGTCGACTTGCCATGGT CTAGAAAGTCTGCGAACCGTTGC 3'** |
| pIVEX2.4d | WP_002969501.1 | LIN2483 | **F: 5' CATCATCATAGCAGCGGCATC ATCGTGGATTCCAATTCCTA 3'** |
| | | LIN2484 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTACCACCTCACCCTGAAACCAA 3'** |
| pIVEX2.4d | WP_002964008.1 | LIN2485 | **F: 5' CATCATCATAGCAGCGGCATC ACGGACAGCAGTGCAAATCC 3'** |
| | | LIN2486 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTACTGCGTCACGAAACAGCTTC 3'** |
| pIVEX2.4d | WP_002964049.1 | LIN2487 | **F: 5' CATCATCATAGCAGCGGCATC AAAAGGATCGAGCGCCTCGT 3'** |
| | | LIN2488 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTATTGGAACTCGAAGATGGAAC 3'** |
| pIVEX2.4d | WP_040120204.1 | LIN2489 | **F: 5' CATCATCATAGCAGCGGCATC CCTGGTCTTTTTAGCTCAAC 3'** |
| | | LIN2490 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTAATTGAAGGTATAGCTGAAAC 3'** |
| pIVEX2.4d | WP_002964749.1 | LIN2491 | **F: 5' CATCATCATAGCAGCGGCATC ACGTTATCGACGCGTATAGC 3'** |
| | | LIN2492 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGGCAGCAACTTGCGATGCTT 3'** |
| pIVEX2.4d | WP_002964288.1 | LIN2493 | **F: 5' CATCATCATAGCAGCGGCATC AGCATTTCCGCATCTCTCGT 3'** |
| | | LIN2494 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGCCCTTGGCGGCTGCGGCCA 3'** |
| pIVEX2.4d | WP_002964289.1 | LIN2495 | **F: 5' CATCATCATAGCAGCGGCATC GCATTGCCTGATTTCAGCAT 3'** |
| | | LIN2496 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCAGGCGCCTTCGGCCGGAGCCT 3'** |
| pIVEX2.4d | WP_002965601.1 | LIN2497 | **F: 5' CATCATCATAGCAGCGGCATC AGCGAGGAAGTGCAAGGCGG 3'** |
| | | LIN2498 | **R':5' CGCTCGAGTCGACTTGCCATGGT TCACGTATTGAGCCCGAGCACGT 3'** |
| pIVEX2.4d | WP_002966987.1 | LIN2499 | **F: 5' CATCATCATAGCAGCGGCATC GCGAAATCCGGCACCCCGCG 3'** |
| | | LIN2500 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTACTGACCGGAAGAGGCCGGAG 3'** |
| pIVEX2.4d | WP_002964995.1 | LIN2501 | **F: 5' CATCATCATAGCAGCGGCATC GCACGCAACAAGATTGCCCT 3'** |
| | | LIN2502 | **R':5' CGCTCGAGTCGACTTGCCATGGT TTATTTCAGCGACGGAGCAATAC 3'** |
| pIVEX2.4d | WP_002964841.1 | LIN2503 | **F: 5' CATCATCATAGCAGCGGCATC CGACTTTTATCCTTGCTTAC 3'** |

| | | LIN2504 | R':5' CGCTCGAGTCGACTTGCCATGGT TCATCTGCTGGTGGCTGCCAGCC 3' |
|---|---|---|---|
| pIVEX2.4d | WP_002964172.1 | LIN2505 | F: 5' CATCATCATAGCAGCGGCATC TTCAATTCGGTACTCGACAC 3' |
| | | LIN2506 | R':5' CGCTCGAGTCGACTTGCCATGGT TTACCCCTCGAACGGTATGTCTA 3' |
| pIVEX2.4d | WP_002965705.1 | LIN2507 | F: 5' CATCATCATAGCAGCGGCATC TTGCGTCTGGTACAGTTCCG 3' |
| | | LIN2508 | R':5' CGCTCGAGTCGACTTGCCATGGT TCACAATGCGCGCACGGTGATTT 3' |

# 4. RESULTS

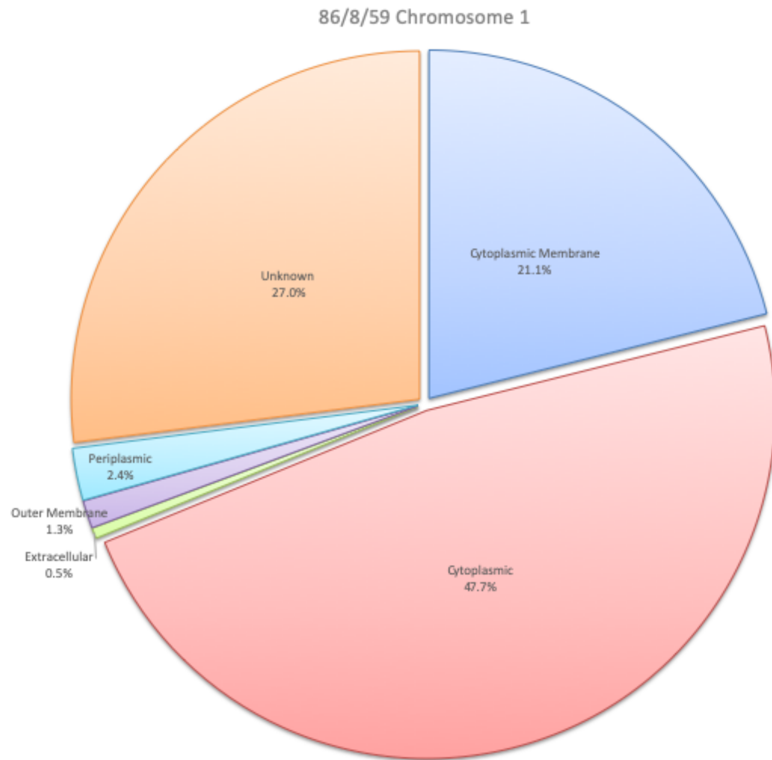
## 4.1 Bioinformatics Analysis of *B. abortus* genome

The thirteen strains of *B. abortus* listed in Table 6 were selected due to the availability of the completed genome sequences in the NCBI bacterial genome database. Ranging from 3.27 to 3.29 Mb in size, the genome sequences of the selected strains were submitted to PSORTb, an online subcellular localization prediction tool, for localization analysis of the proteins encoded by all the ORFs. Figure 6 shows a breakdown of the proteins encoded by each chromosome of strain 86/8/59 according to protein subcellular localization. This strain is available for use in the study and thus its PSORTb result presented here in Figure 6.

After consolidating the protein subcellular localization data from the thirteen strains and identifying common proteins in Excel, a total of 137 unique proteins were identified as being outer membrane. In this case, a unique protein is considered a protein with a single NCBI accession number, regardless of which strain or how many strains it is present in. Of these proteins, 42 proteins were selected as protein candidates as they are conserved over seven or more strains, where at least one strain was 86/8/59 (Table 7).

PSORTb analysis resulted in numerous proteins of unknown localization (Figure 6). The candidate list was expanded to include twenty proteins of unknown localization with the highest strain coverage (Table 8).

Throughout the years, a number of *B. abortus* proteins have been reported in literature as antigenic proteins(114, 132). Thirty-eight such proteins were also added to the candidate list (Table 9). In total, 100 proteins were selected as candidates for assessing their potential as diagnostic antigens.



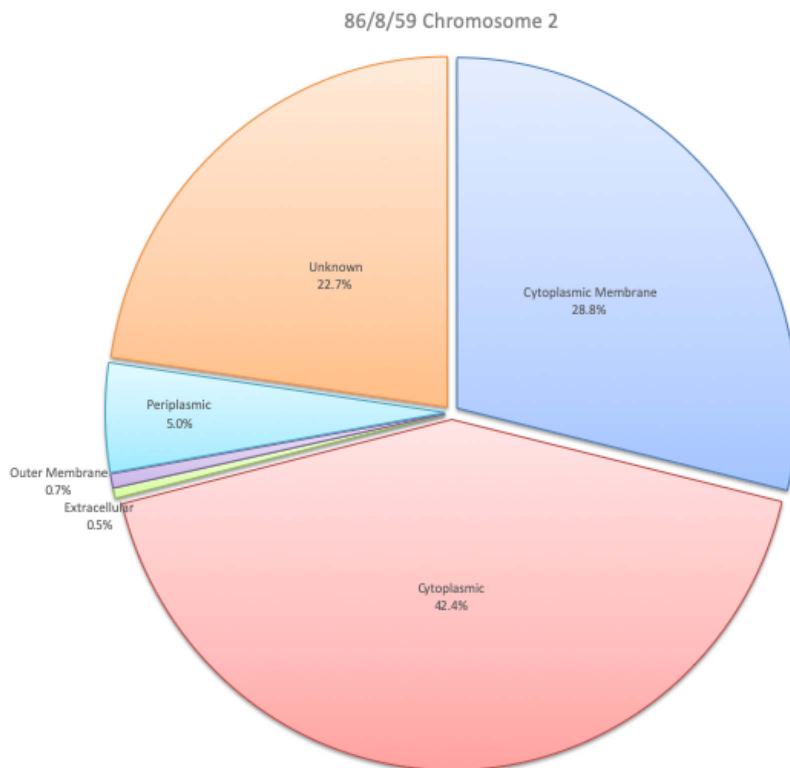




*Figure 6. Localization distribution of B. abortus 86/8/59 proteins for each chromosome as per PSORTb.*

*Table 6. Thirteen Brucella abortus strains chosen for PSORTb analysis, identified by strain ID and accession number. Strains were chosen because their whole genome was sequenced.*

| Strain ID | Chromosome | Accession Number |
|---|---|---|
| 9-941 | 1 | NC_006932.1 |
| | 2 | NC_006933.1 |
| 63/75 | 1 & 2 | NZ_CP007662.1 |
| 86-8-59 | 1 | NZ_CP007765.1 |
| | 2 | NZ_CP007764.1 |
| 870 | 1 | NZ_CP007709.1 |
| | 2 | NZ_CP007710.1 |
| 2308 | 1 | NC_007618.1 |
| | 2 | NC_007624.1 |
| A13334 | 1 | CP003176.1 |
| | 2 | CP003177.1 |
| BAB8416 | 1 | NZ_CP008774.1 |
| | 2 | NZ_CP008775.1 |
| BDW | 1 | NZ_CP007681.1 |
| | 2 | NZ_CP007680.1 |
| BER | 1 | NZ_CP007682.1 |
| | 2 | NZ_CP007683.1 |
| BFY | 1 | NZ_CP007738.1 |
| | 2 | NZ_CP007737.1 |
| C68 | 1 | NZ_CP007705.1 |
| | 2 | NZ_CP007706.1 |
| NCTC 10505 | 1 | NZ_CP007700.1 |
| | 2 | NZ_CP007701.1 |
| S19 | 1 | NC_010742.1 |
| | 2 | NC_010740.1 |

*Table 7. Forty-two proteins conserved in seven or more strains. Proteins are described by chromosome, location, and protein sequence ID.*

| Number of Strains Covered | Chromosome | Location | Protein Sequence ID |
|---|---|---|---|
| 13 | 1 | Extracellular | WP_002971034.1 |
| | | Outer Membrane | WP_002964402.1 |
| | | | WP_002964530.1 |
| | | | WP_002965367.1 |
| | | | WP_002965368.1 |
| | 2 | Extracellular | WP_002966649.1 |
| | | Outer Membrane | WP_002966502.1 |
| 12 | 1 | Extracellular | WP_002963836.1 |
| | | | WP_002964637.1 |
| | | Outer Membrane | WP_002963597.1 |
| | 2 | Extracellular | WP_002966373.1 |
| 11 | 1 | Extracellular | WP_002963986.1 |
| | | | WP_002965213.1 |
| | | Outer Membrane | WP_002964019.1 |
| | | | WP_002964282.1 |
| | | | WP_002964719.1 |
| | 2 | Extracellular | WP_002966636.1 |
| | | Outer Membrane | WP_002965726.1 |
| | | | WP_002971189.1 |
| 10 | 1 | Outer Membrane | WP_002963844.1 |
| | | | WP_002964008.1 |
| | | | WP_002964666.1 |
| | | | WP_002964782.1 |
| | | | WP_002964998.1 |
| | | | WP_002969994.1 |
| | | | WP_002971512.1 |
| | 2 | Extracellular | WP_002966644.1 |
| | | Outer Membrane | WP_002966591.1 |
| 9 | 1 | Outer Membrane | WP_002964462.1 |
| | 2 | Extracellular | WP_002965683.1 |
| 8 | 1 | Extracellular | WP_002965076.1 |
| | | Outer Membrane | WP_002964049.1 |
| | | | WP_002966947.1 |
| | | | WP_025197938.1 |
| 7 | 1 | Extracellular | WP_002964958.1 |
| | | | WP_002965376.1 |
| | | Outer Membrane | WP_002970988.1 |
| 6 | 1 | Extracellular | WP_002966987.1 |
| | | | WP_002966988.1 |
| | | | WP_002967016.1 |
| | | Outer Membrane | WP_002969883.1 |
| | 2 | Outer Membrane | WP_002967131.1 |

*Table 8. Twenty proteins of unknown localization conserved in 10 strains. Proteins are described by chromosome, location, and protein sequence ID.*

| Number of Strains Covered | Chromosome | Location | Protein Sequence ID |
|---|---|---|---|
| 10 | 1 | Unknown | WP_002963384.1 |
| | | | WP_002963464.1 |
| | | | WP_002963473.1 |
| | | | WP_002963488.1 |
| | | | WP_002963504.1 |
| | | | WP_002963512.1 |
| | | | WP_002963525.1 |
| | | | WP_002963529.1 |
| | | | WP_002963545.1 |
| | | | WP_002963546.1 |
| | | | WP_002963553.1 |
| | | | WP_002963554.1 |
| | | | WP_002963558.1 |
| | | | WP_002963567.1 |
| | | | WP_002963581.1 |
| | | | WP_002963582.1 |
| | | | WP_002963584.1 |
| | | | WP_002963592.1 |
| | | | WP_002963624.1 |
| | | | WP_002963635.1 |

*Table 9. Thirty-eight proteins selected from literature. Proteins are described by chromosome, location, and protein sequence ID.*

| Chromosome | Location | Protein Sequence ID |
|---|---|---|
| 1 | unknown | WP_002964995.1 |
| | | WP_002970984.1 |
| | | WP_002971551.1 |
| | Cytoplasmic | WP_002964749.1 |
| | | WP_002964288.1 |
| | | WP_002964289.1 |
| | | WP_002965053.1 |
| | | WP_002964601.1 |
| | | WP_002963897.1 |
| | | WP_002964172.1 |
| | | WP_002965266.1 |
| | | WP_002963577.1 |
| | | WP_002967114.1 |
| | | WP_002964293.1 |
| | | WP_002965534.1 |
| | | WP_002966968.1 |
| | | WP_002968051.1 |
| | | WP_002966970.1 |
| | | WP_002965390.1 |
| | | WP_002963466.1 |
| | | WP_002963831.1 |
| | | WP_002969557.1 |
| | | WP_002963876.1 |
| | Periplasmic | WP_002964841.1 |
| | | WP_002963760.1 |
| 2 | Cytoplasmic | WP_002965601.1 |
| | | WP_002966387.1 |
| | | WP_002965705.1 |
| | | WP_002965779.1 |
| | | WP_002965780.1 |
| | | WP_002965788.1 |
| | | WP_002968536.1 |
| | | WP_002965581.1 |
| | | WP_002972147.1 |
| | | WP_002966033.1 |
| | Periplasmic | WP_002966556.1 |
| | | WP_002967303.1 |
| | | WP_002966589.1 |

## 4.2 Generation of Protein Expression Constructs

### 4.2.1 Creation of Linearized Plasmid Vector

Plasmid vectors pIVEX2.3d and pIVEX2.4d were linearized by restriction enzyme digestion at the multiple cloning site (MCS). As shown in Figure 7, a faint secondary band is observed with the NotIHF-digested vectors, whereas *Nde*I digestion has the desired single band close to the calculated linear sizes of 3560 bp for pIVEX2.3d and 3583 bp for pIVEX2.4d. When comparing the size of the secondary product to that of the product in lane 3, that secondary band may indicate the presence of undigested vector. Thus, future digestion would continue forward with *Nde*I. Linearized plasmids were further amplified by PCR and confirmed by PCR as a single defined band when analyzed through agarose gel electrophoresis.

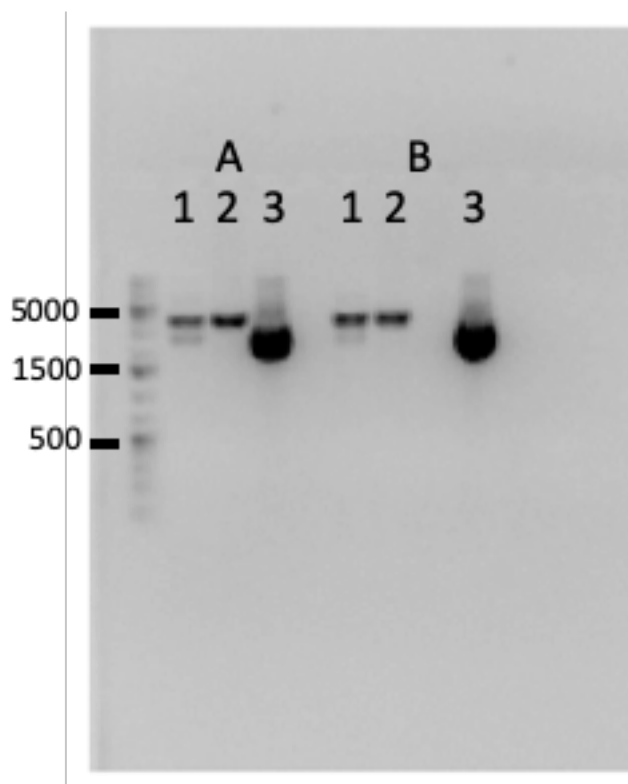



*Figure 7. Agarose gel electrophoresis of vectors: (A) pIVEX2.3d and (B) pIVEX2.4d. Wells are as follows: (1) NotIHF-digested, (2) NdeI digested, and (3) undigested, circularized vector. Molecular ladder used is GeneRuler 1 kb plus DNA ladder (Invitrogen).*

### 4.2.2 Synthesis by PCR of ORFs Coding for Protein Candidates

The ORFs coding for the selected protein candidates were synthesized by PCR from the genomic sequence of heat-inactivated strain 86/8/59 using gene-specific primers (Table 4 and Table 5). These primers were designed for cloning the target ORFs into the pIVEX2.3d or pIVEX2.4d expression vectors. Of 100 target ORFs, 95 were successfully synthesized by PCR. Only five candidates were unsuccessfully amplified, either failing to display a single band or having multiple bands of similar sizes. Figure 8 shows the successful synthesis of one of the ORFs, the *BCSP31* gene. The PCR method was tested with three

different *B. abortus* strains, resulting in the amplification of a correctly-sized DNA fragment, albeit with some other nonspecific products, including the low molecular weight primer dimers. The ORFs of correct sizes were recovered through the excision of the desired band followed by the use of gel extraction. This process also helped remove the other minor PCR products.

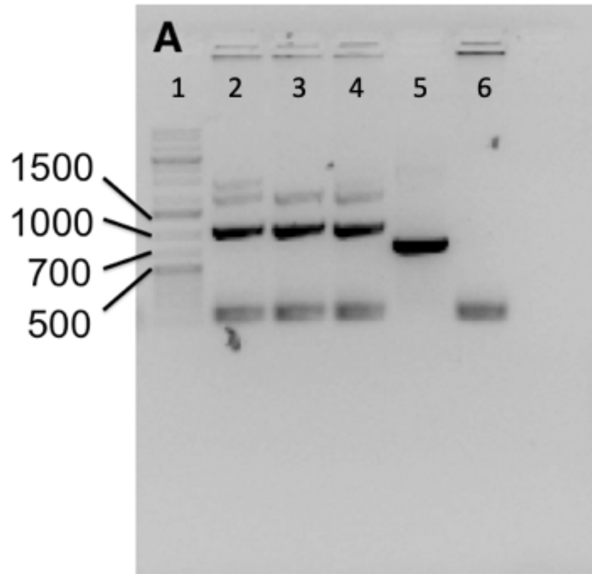



*Figure 8. Agarose gel electrophoresis analysis of PCR products. Successful synthesis of a candidate (BCSP31 gene) ORF by PCR amplification from three B. abortus strains provided by CFIA. (1) 5 µL of GeneRuler 1 kb plus DNA ladder (Invitrogen); BCSP31 isolated from (2) strain 544 (3) biovar 86/8/59, (4) Strain 19; (5) PCR positive control (GFPuv), and (6) negative control.*

### 4.2.3 Cloning of Target ORFs into Expression Vectors

The co-transformation of *E. coli* DH5α with each of the 95 target ORFs and the linearized plasmid vector pIVEX2.3d or pIVEX2.4d led to the formation of 91 recombinant plasmids. The presence of a correct insert in the recombinant plasmids was confirmed by PCR with the T7 promoter and T7 terminator primers, which is exemplified in Figure 9. As shown in Figure 9, a recombinant plasmid shows the size of the PCR product close to the size of the ORF plus the size of an approximately 250 to 260 bp DNA fragment, which represents the size of the PCR product derived from an empty plasmid vector. All recombinant plasmids were sequenced to verify the correct ORF inserts.

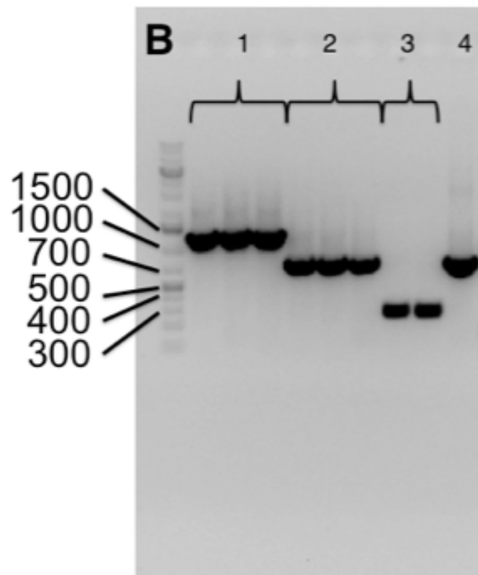



*Figure 9. Agarose gel electrophoresis analysis of PCR products. Identification of recombinant plasmids using colony PCR: (1) successful pIVEX2.3d-BCSP31; (2) successful pIVEX2.3d-VirB12 and; (3) empty pIVEX2.3d negative control; (4) PCR positive control – GFPuv.*

## 4.3 Generation of Recombinant Proteins by *In Vitro* Transcription/Translation

### 4.3.1 *In Vitro* Transcription/Translation

The 91 successful recombinants underwent *in vitro* transcription/translation in order to complete the generation of corresponding recombinant proteins. The initial test protocol of the entire workflow starting from 4.2.2 (Synthesis by PCR of ORFs Coding for Protein Candidates) was performed with the GFPuv gene. The presence of fluorescence (Figure 10) is a sufficient indicator for the successful expression of GFPuv protein with proper folding *in vitro* in lieu of detection through specific antibodies, thereby eliminating an extra step during the protein expression optimization. Also tested during optimization with GFPuv was the amount of recombinant plasmid that was to be used. An expression attempt with 250 ng of recombinant plasmid vector did not yield fluorescence when compared to the kit's positive control (Figure 10A vs 10D), but with 100 ng of the recombinant plasmid appeared to be successful in the protein expression (Figure 10B&C).

*Figure 10. Completed in vitro transcription/translation of GFPuv recombinant protein. (A) 2:1 ratio of GFPuv:pIVEX2.4d (250 ng vector), (B) 2:1 ratio GFPuv:pIVEX2.3d (100 ng vector) (C) 2:1 ratio of GFPuv:pVIEX2.4d (100ng vector), (D) Positive GFP control from kit, (E) Negative control (water).*

### 4.3.2 Detection of Protein Expression using Western Blot

Recombinant proteins expressed from the ORF candidates cloned into pIVEX2.3d or pIVEX2.4d did not emit a fluorescence. As such, expression of these proteins containing a polyhistidine tag at the C- or N-terminus was detected with Western blots probed with anti-His monoclonal antibodies. As demonstrated in Figure 11, successful protein expression leads to a distinguishable band, with the visually-confirmed GFPuv protein acting as a positive control on the Western blot. Two *B. abortus* proteins, BCSP31 and VirB12, were used in the process optimization. BCSP31, a 31 kDa protein in size, was detected by Western blot with a band of the correct size. In contrast, the VirB12 failed to express. Failure to express with the initial pIVEX2.3d vector triggered a second attempt of cloning the ORF into pIVEX2.4d. The use of two vectors that express a protein with a polyhistidine tag at different termini eliminates the possible interference of protein expression by the tag location. Across the two vectors, there was a set of 67 successfully formed proteins, with 24 candidates failing to express.

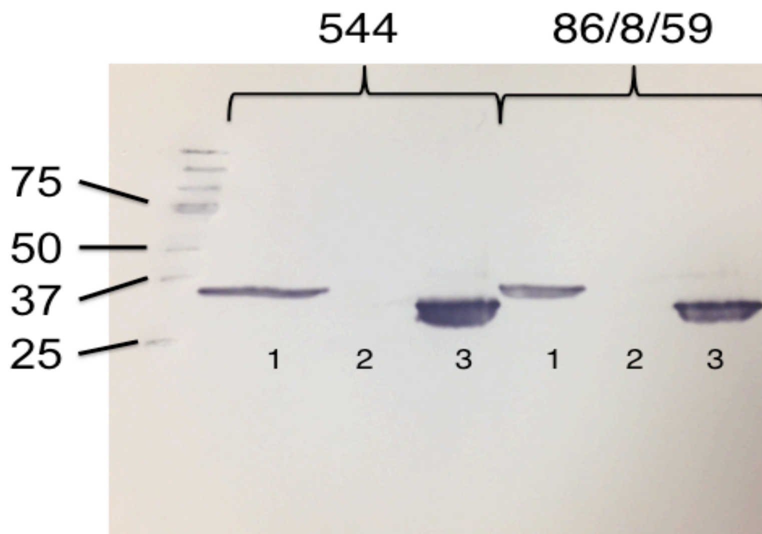
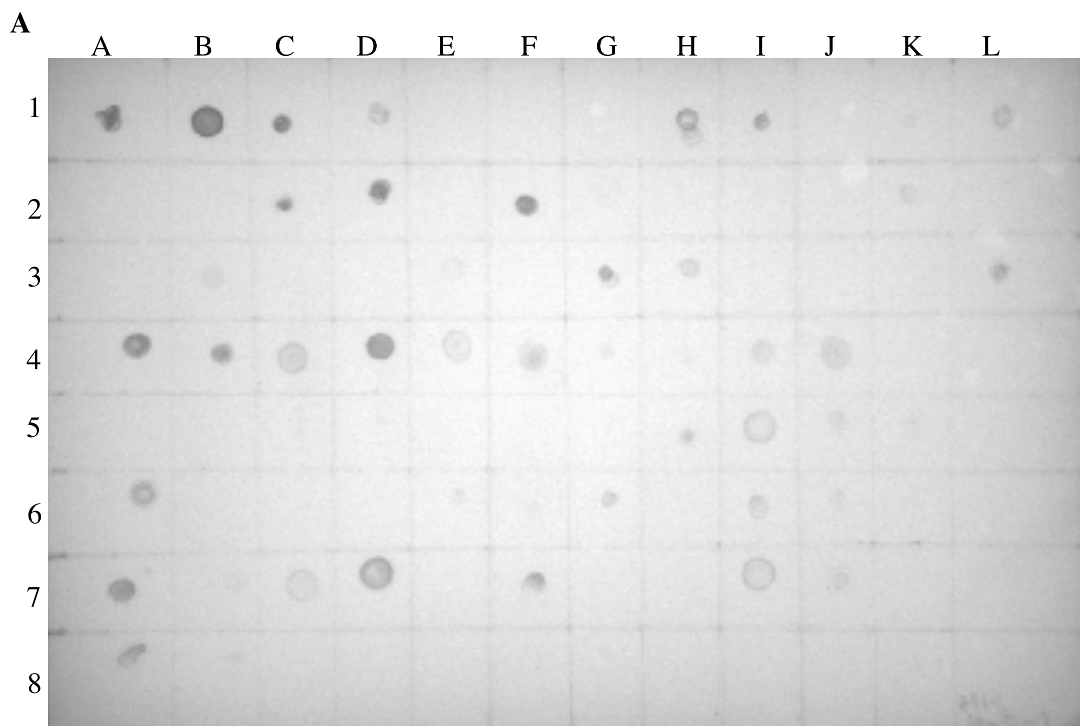



*Figure 11. Western blot analysis of in vitro expressed proteins. Two target proteins from each of the 2 biovar test strains, expressed using an RTS 100 E. coli HY kit, were probed with an anti-His Mab. 1) Successful BCSP31; 2) Unsuccessful VirB12; 3) Positive Control – GFPuv*

#### 4.3.3 Assessment of Protein Expression using Quantity One Software

Initial screening of the 67 unique proteins expressed from the cloned ORFs was done via dot blot assay in order to detect the presence of His-tags, as done in the IVTT confirmation step using Western blots. Numerous ORFs were tested in both vectors. All proteins were screened on a single membrane in order to determine relative concentrations of each candidate protein (Figure 12).




**B**

| | A | B | C | D | E | F | G | H | I | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1-01 | 18-02 | 1-03 | 18-03 | 2-02 | 2-03 | | 2-04 | 2-07 | 2-08 | 2-09 | 19-11 |
| 2 | 5-01 | 20-01 | 5-02 | 5-04 | 11-01 | 21-01 | 11-02 | 21-03 | 11-04 | 21-04 | 11-05 | 21-05 |
| 3 | 11-06 | 11-07 | 21-07 | 21-08 | 21-09 | 21-10 | 22-01 | 22-04 | 12-06 | 22-06 | 12-07 | 12-09 |
| 4 | 23-01 | 23-02 | 14-03 | 14-06 | 14-07 | 14-08 | 14-09 | 14-10 | 23-10 | 14-11 | 3-01 | 3-03 |
| 5 | 25-01 | 23-02 | 13-04 | 27-01 | 27-02 | 27-03 | 15-05 | 15-07 | 15-10 | 15-12 | 15-13 | 15-14 |
| 6 | 15-15 | 4-01 | 4-02 | 4-03 | 16-01 | 30-01 | 30-02 | 8-01 | 32-01 | 33-01 | 9-01 | 34-01 |
| 7 | 9-02 | 34-02 | 9-04 | 34-04 | 10-01 | 10-03 | 10-04 | 10-05 | 10-06 | 35-07 | 35-08 | 35-09 |
| 8 | BCSP31 | 2-10 | 12-05 | 23-05 | 13-05 | 27-06 | 7-01 | 10-10 | pIVEX 2.4D | GFP | LPS | negative |

*Figure 12. Dot blot assay of the candidate proteins, probing for the His-tags of each protein (A). The candidates were tested in the order as shown in (B). Candidates were assessed for presence of His-tag using (anti-His) mAbs. 1 µL of each sample was dotted onto the membrane.*

In order to determine the relative concentrations, the Quantity One software was used. The program allows for the measure of relative densities of each candidate (Figure 13), independent of circle size. Candidates were normalized against the strongest reaction (i.e., highest volume density), the candidate labelled U2 in Figure 13, also referred to as candidate 18-02.

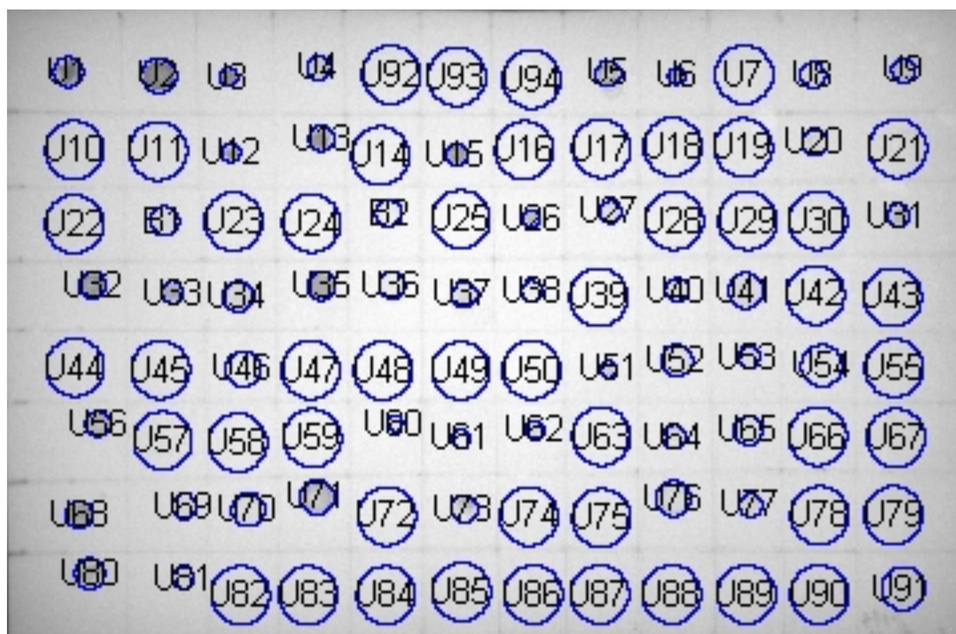


*Figure 13. Volume Analysis in Quantity One. Each U circle (U1 to U 94) represents a candidate, with circles B1 and B2 used for background calculations.*

## 4.4 Reactivity of Recombinant Proteins with Sera from *B. abortus*-infected Cattle

A panel of sera was tested for the reactivity with LPS-coated plates in an ELISA protocol, using a dilution series for each serum, as shown in Table 10A. The dilution series was required, as even at a 1/100 dilution, most of the serum resulted in an “OVERFLW” message (Table 10B), indicating an absorbance over the machine’s maximum readings at $OD_{414}$. These dilutions also helped to show that prozone effect was not an issue, as the signal seems to decrease as the serum is serially diluted. A prozone effect, where signal is lower than expected, would be the result of antibody levels being too high. In this case, performing dilutions would show a spike in signal at a lower dilution, as the binding of the antibody would then match the analyte level(133).

Because the expressed candidate proteins were made using an *E. coli* lysate system, cross-reactivity of the serum with any *E. coli* components may lead to a false-positive. Knowing this, a control dot blot was performed, which revealed cross-reactions of a C++ serum, a strong positive reference serum, with known negative samples, including the protein samples expressed from empty pIVEX vectors and the lysate itself (Figure 14). S22, a moderately reactive positive serum, was chosen to be adsorbed, visually demonstrated a reduction in binding when compared to its native state (Figure 15). A similar phenomenon was observed the pre-adsorption of a highly reactive positive serum (Figure 16). A highly reactive positive serum is more likely to come from a cow demonstrating more severe symptoms due to the presence of more bacteria.

*Table 10. Serum was serially diluted and applied to an LPS-coated plate in the order of (A). Readings of the panel of bovine-infected serum as the primary antibody at an OD of 414 are shown in (B). Blue colouring in table indicates wells that obtained a reading, whereas other wells gave an "OVERFLW" response.*

**A**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 - 1/100 | 1 - 1/200 | 1 - 1/400 | 1 - 1/800 | 1 - 1/1600 | 3 - 1/100 | 3 - 1/200 | 3 - 1/400 | 3 - 1/800 | 3 - 1/1600 | pos 1/100 | part pos 1/100 |
| B | 6 - 1/100 | 6 - 1/200 | 6 - 1/400 | 6 - 1/800 | 6 - 1/1600 | 9 -1/100 | 9 -1/200 | 9 -1/400 | 9 -1/800 | 9 - 1/1600 | pos 1/200 | part pos 1/200 |
| C | 10 -1/100 | 10 - 1/200 | 10-1/400 | 10-1/800 | 10 - 1/1600 | 11 - 1/100 | 11 - 1/200 | 11 - 1/400 | 11 - 1/800 | 11 - 1/1600 | pos 1/400 | part pos 1/400 |
| D | 16 - 1/100 | 16 - 1/200 | 16 - 1/400 | 16 - 1/800 | 16 - 1/1600 | 18 - 1/100 | 18 - 1/200 | 18 - 1/400 | 18 - 1/800 | 18 - 1/1600 | pos 1/800 | part pos 1/800 |
| E | 19 - 1/100 | 19 - 1/200 | 19 - 1/400 | 19 - 1/800 | 19 - 1/1600 | 21 - 1/100 | 21 -1/200 | 21 -1/400 | 21 - 1/800 | 21 - 1/1600 | pos 1/1600 | part pos 1/1600 |
| F | 22 - 1/100 | 22 - 1/200 | 22 - 1/400 | 22 - 1/800 | 22 - 1/1600 | 23 - 1/100 | 23 - 1/200 | 23 - 1/400 | 21 - 1/800 | 23 - 1/1600 | C++ 1/100 | C++ 1/200 |
| G | 27 - 1/100 | 27 - 1/200 | 27 - 1/400 | 27 - 1/800 | 27 - 1/1600 | 30 - 1/100 | 30 - 1/200 | 30 - 1/400 | 21 - 1/800 | 30 - 1/1600 | C++ 1/400 | C++ 1/800 |
| H | 31 - 1/100 | 31 - 1/200 | 31 - 1/400 | 31 - 1/800 | 31 - 1/1600 | 33 - 1/100 | 33 - 1/200 | 33 - 1/400 | 33 - 1/800 | 33 - 1/1600 | C++ 1/1600 | neg |

**B**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | 2.283 | 2.277 | 1.812 | 1.89 | 2.187 | OVRFLW | 2.49 |
| B | OVRFLW | 2.588 | 2.149 | 1.731 | 1.217 | OVRFLW | OVRFLW | OVRFLW | OVRFLW | 2.515 | OVRFLW | 2.708 |
| C | OVRFLW | 2.872 | 2.476 | 2.202 | 2.061 | 1.768 | 1.656 | 1.421 | 0.929 | 0.745 | OVRFLW | 2.75 |
| D | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | 2.658 | 2.231 | 2.373 | OVRFLW | OVRFLW |
| E | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | 2.791 | 1.903 | OVRFLW | 2.579 |
| F | OVRFLW | 2.493 | 1.772 | 1.597 | 1.395 | 1.785 | 1.884 | 1.632 | 1.99 | 1.256 | OVRFLW | OVRFLW |
| G | 1.795 | 1.54 | 1.829 | 1.55 | 0.937 | 1.214 | 1.226 | 1.321 | 1.07 | 0.702 | OVRFLW | OVRFLW |
| H | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | OVRFLW | 2.096 | OVRFLW | 0.075 |

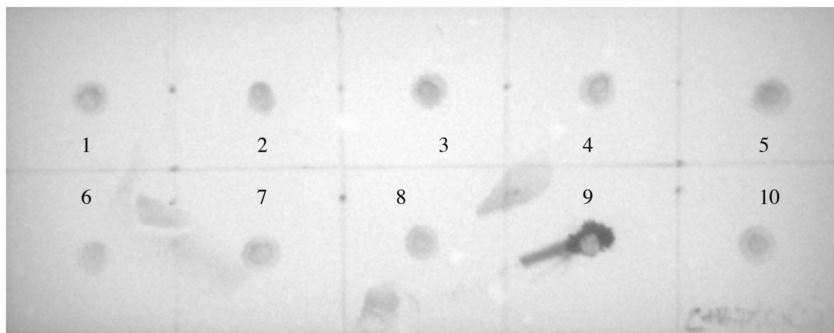



*Figure 14. Dot blot of candidates and controls using vaccinated bovine C++ serum. The detection of expressed proteins used vaccinated bovine C++ serum as the primary antibody at a 1/250 dilution. The secondary antibody was an HRP-derived goat anti-bovine antibody. Candidates were (1)2-08, (2) 34-01, (3) 34-04, (4) 10-06, and (5) BCSP31. Controls included were (6) pIVEX2.3d, (7) pIVEX2.4d, (8) GFP, (9) LPS positive control, and (10) lysate negative control.*

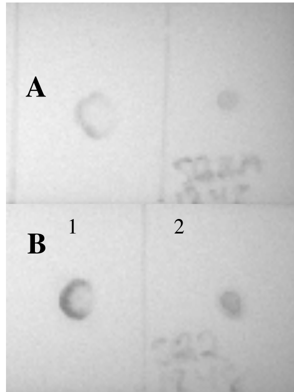



*Figure 15. Dot blot of controls using moderately reactive bovine serum. A comparison of detection using (A) Serum 22 pre-adsorbed with E. coli vs (B) Unadsorbed serum 22 as primary antibodies at a 1/250 dilution. An HRP-derived goat anti-bovine secondary antibody was used for detection. Test controls were (1) LPS positive control, and (2) E. coli lysate negative control.*

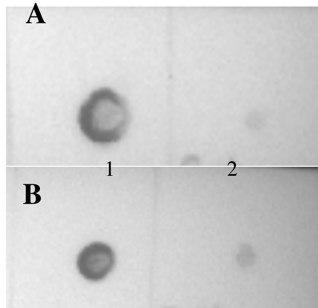



*Figure 16. Dot blot of controls using positive bovine serum. A comparison of detection using (A) Positive serum pre-adsorbed with E. coli vs (B) Unadsorbed positive serum as primary antibodies at a 1/250 dilution. An HRP-derived goat anti-bovine secondary antibody was used for detection. Test controls were (1) LPS positive control, and (2) E. coli lysate negative control.*

| | A | B | C | D | E | F | G | H | I | J | K | L | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1-01 | 18-02 | 1-03 | 18-03 | 2-02 | 2-03 | | 2-04 | 2-07 | 2-08 | 2-09 | 19-11 | 1 uL |
| 2 | 5-01 | 20-01 | 5-02 | 5-04 | 11-01 | 21-01 | 11-02 | 21-03 | 11-04 | 21-04 | 11-05 | 21-05 | 2 uL |
| 3 | 11-06 | 11-07 | 21-07 | 21-08 | 21-09 | 21-10 | 22-01 | 22-04 | 12-06 | 22-06 | 12-07 | 12-09 | 3 uL |
| 4 | 23-01 | 23-02 | 14-03 | 14-06 | 14-07 | 14-08 | 14-09 | 14-10 | 23-10 | 14-11 | 3-01 | 3-03 | 4 uL |
| 5 | 25-01 | 23-02 | 13-04 | 27-01 | 27-02 | 27-03 | 15-05 | 15-07 | 15-10 | 15-12 | 15-13 | 15-14 | 5 uL |
| 6 | 15-15 | 4-01 | 4-02 | 4-03 | 16-01 | 30-01 | 30-02 | 8-01 | 32-01 | 33-01 | 9-01 | 34-01 | 6 uL |
| 7 | 9-02 | 34-02 | 9-04 | 34-04 | 10-01 | 10-03 | 10-04 | 10-05 | 10-06 | 35-07 | 35-08 | 35-09 | 7 uL |
| 8 | BCSP31 | 2-10 | 12-05 | 23-05 | 13-05 | 27-06 | 7-01 | 10-10 | pIVEX 2.4I | GFP | LPS | negative | 8 uL |

*Figure 17. Amounts of each candidate protein to use after considering relative interaction, as indicated by the volume to colour legend on the right of the figure. The 18-02 candidate (B1) had the highest adjusted volume and was the candidate against which the other candidates were normalized.*

### 4.4.1 Protein Array Analysis of the Reactivity of Protein Candidates with Sera

Upon normalizing the concentrations of each recombinant protein, they were dotted at the calculated volumes shown in Figure 17 and probed with the three sera: S+ (Figure 18), S22 (Figure 19), and pre-adsorbed S22 (Figure 20).

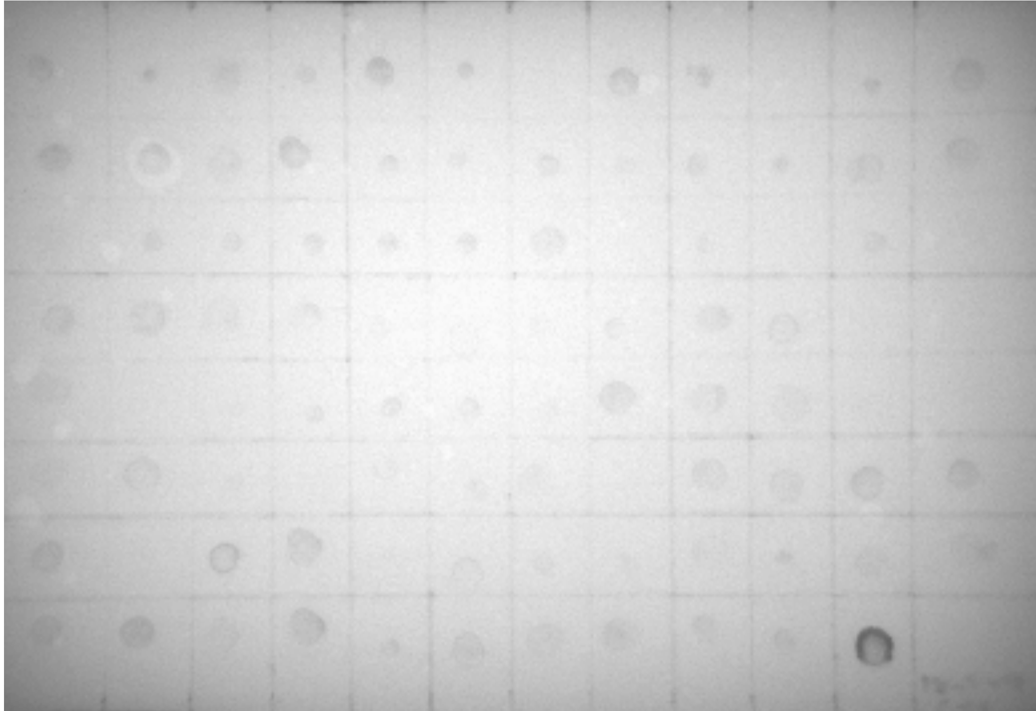


*Figure 18. Dot blot assay of antigenic candidates, using S+ serum. Candidates, as identified in Figure 12B, were assessed for antigenicity against B. abortus antibodies using S+ serum. Candidates were dotted at volumes indicated in Figure 17.*

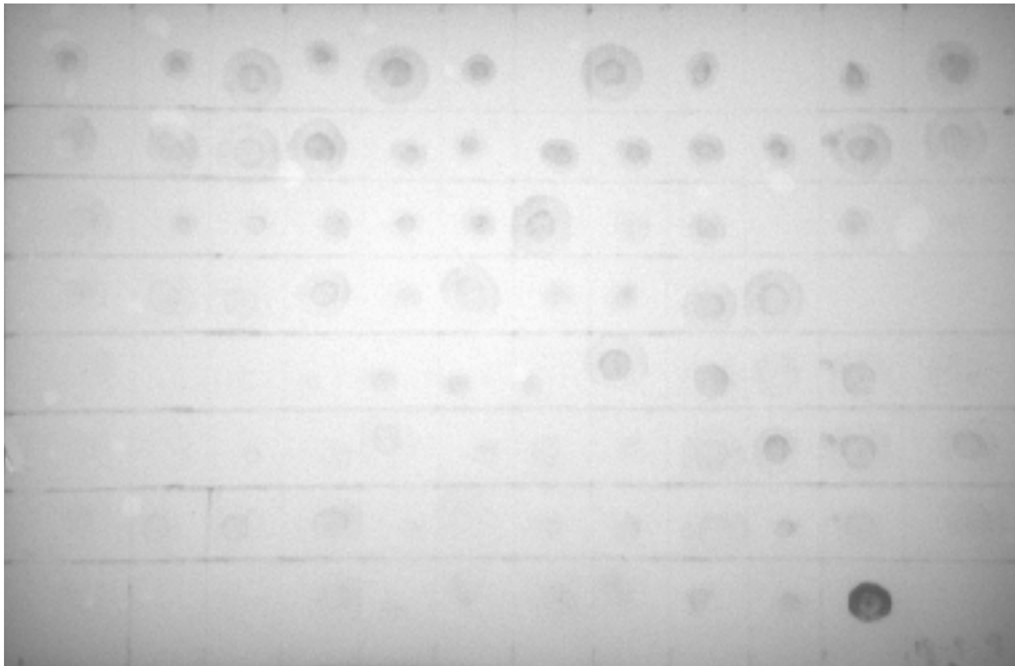


*Figure 19. Dot blot assay of antigenic candidates, using S22 serum. Candidates, as identified in Figure 12B, were assessed for antigenicity against B. abortus antibodies using S22 serum. Candidates were dotted at volumes indicated in Figure 17.*

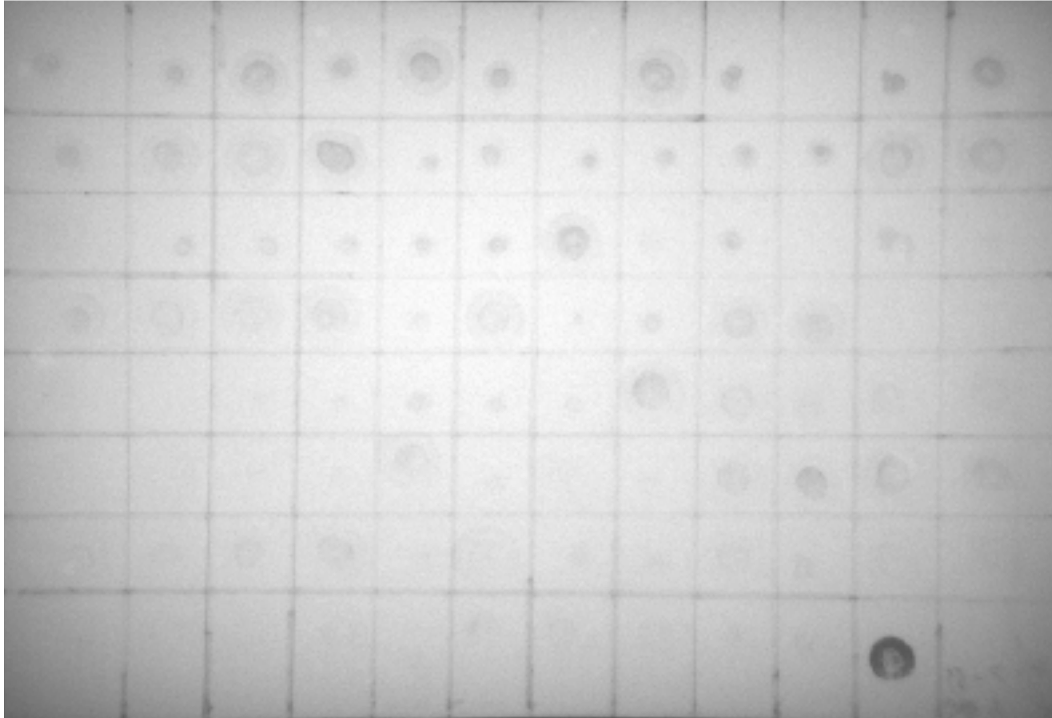


*Figure 20. Dot blot assay of antigenic candidates, using pre-adsorbed S22 serum. Candidates, as identified in Figure 12B, were assessed for antigenicity against B. abortus antibodies using pre-adsorbed S22 serum. Candidates were dotted at volumes indicated in Figure 17.*

Using the Quantity One software as described in Materials and Methods, the obtained densities were converted to a percentage reactivity, with the positive LPS control acting as the maximum reactivity (100%). Protein candidates that demonstrated a reactivity higher than the truncated mean percentage reactivity for each test was considered to have a positive antibody-antigen interaction. The twenty-four protein candidates that were positive for the three antibody-containing sera (S+, S22, and pre-adsorbed S22) were selected for another two rounds of protein array analysis assay. The threshold was a personal choice and as such, the threshold was also decreased to 40% of the truncated population. This expanded the options to include ten more candidates for a total of thirty-four candidates to be tested in a second and third round (Table 11).

*Table 11. Candidates selected for secondary and tertiary testing based on the number of serum conditions the candidate interactions with.*

| Threshold Condition | Candidate | Adjusted Sera Condition |
|---|---|---|
| 50% Threshold | 1-01 | N/A |
| | 1-03 | |
| | 2-02 | |
| | 2-04 | |
| | 2-07 | |
| | 19-11 | |
| | 5-01 | |
| | 5-02 | |
| | 5-04 | |
| | 11-05 | |
| | 21-05 | |
| | 22-01 | |
| | 23-02 | |
| | 23-10 | |
| | 14-11 | |
| | 15-07 | |
| | 15-10 | |
| | 32-01 | |
| | 33-01 | |
| | 9-01 | |
| | 34-01 | |
| | 9-04 | |
| | 34-04 | |
| | 10-06 | |
| 40% Threshold | 2-03 | S+ |
| | 11-01 | |
| | 21-01 | |
| | 11-02 | |
| | 11-04 | |
| | 21-08 | |
| | 21-09 | |
| | 14-06 | |
| | 14-10 | |
| | 27-02 | S+ and S1 |

From the initial list of candidates that underwent preliminary screening, thirty-four proteins were screened for a total of three tests per serum condition, as indicated in Table 11. These tests assessed the antigenicity of each candidate, which were used as antigens to react with the serum's antibodies. For each protein array, LPS acted as a positive control antigen and *E. coli* lysate the negative control. This was done a total of three times for each sera condition (Table 12).

The most promising candidates were those (5-02, 11-05, 21-05, 14-11, 33-01, 9-04, and 34-04) that met the truncated mean for proportional antibody-antigen reactivity threshold across all nine membranes. When the threshold was decreased to 40%, as had been done during the primary screening,

five other proteins (23-02, 23-10, 15-10, 34-01, and 21-01) demonstrated positive reaction with sera across the nine tests. Antigenic candidates are summarized in Table 13.

*Table 12. Final results for the screened candidates across the three gels for each serum condition. Green indicates candidates that met the 50% proportional interaction threshold. Orange represents candidates that met the 40% proportional interaction threshold.*

| | | | S+ | | | S1 | | | S1 pre-adsorbed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Row | Column | Candidates | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| A | 1 | 1-01 | | | | | | | | | |
| | 2 | 1-03 | | | | | | | | | |
| | 3 | 2-02 | | | | | | | | | |
| | 4 | 2-04 | | | | | | | | | |
| | 5 | 2-07 | | | | | | | | | |
| | 6 | 19-11 | | | | | | | | | |
| B | 1 | 5-01 | | | | | | | | | |
| | 2 | 5-02 | | | | | | | | | |
| | 3 | 5-04 | | | | | | | | | |
| | 4 | 11-05 | | | | | | | | | |
| | 5 | 21-05 | | | | | | | | | |
| | 6 | 22-01 | | | | | | | | | |
| C | 1 | 23-02 | | | | | | | | | |
| | 2 | 23-10 | | | | | | | | | |
| | 3 | 14-11 | | | | | | | | | |
| | 4 | 15-07 | | | | | | | | | |
| | 5 | 15-10 | | | | | | | | | |
| | 6 | 32-01 | | | | | | | | | |
| D | 1 | 33-01 | | | | | | | | | |
| | 2 | 9-01 | | | | | | | | | |
| | 3 | 34-01 | | | | | | | | | |
| | 4 | 9-04 | | | | | | | | | |
| | 5 | 34-04 | | | | | | | | | |
| | 6 | 10-06 | | | | | | | | | |
| E | 1 | 2-03 | | | | | | | | | |
| | 2 | 11-01 | | | | | | | | | |
| | 3 | 21-01 | | | | | | | | | |
| | 4 | 11-02 | | | | | | | | | |
| | 5 | 11-04 | | | | | | | | | |
| | 6 | 21-08 | | | | | | | | | |
| F | 1 | 21-09 | | | | | | | | | |
| | 2 | 14-06 | | | | | | | | | |
| | 3 | 14-10 | | | | | | | | | |
| | 4 | 27-02 | | | | | | | | | |
| | 5 | LPS | | | | | | | | | |
| | 6 | Negative Control | | | | | | | | | |

*Table 13. Summary of positive candidates. Location of protein is the PSORTb-returned final localization. The candidates selected from literature are identified below. When examined in PSORTb, they were identified as being cytoplasmic.*

| Candidate | Protein Accession Number | Location | 50% Threshold | 40% Threshold | Total Positive Assays |
|---|---|---|---|---|---|
| 5-02 | WP_002966947.1 | Outer Membrane | 9 | 0 | 9 |
| 11-05 | WP_002963529.1 | Unknown | | | |
| 21-05 | WP_002963529.1 | Unknown | | | |
| 14-11 | WP_002965780.1 | Cytoplasmic | | | |
| 33-01 | WP_040120204.1 | Outer Membrane | | | |
| 9-04 | WP_002965601.1 | Literature | | | |
| 34-04 | WP_002965601.1 | Literature | | | |
| 23-02 | WP_002967114.1 | Cytoplasmic | 8 | 1 | 9 |
| 23-10 | WP_002965779.1 | Unknown | | | |
| 15-10 | WP_002963831.1 | Cytoplasmic | 7 | 2 | 9 |
| 34-01 | WP_002964749.1 | Literature | 6 | 3 | 9 |
| 21-01 | WP_002963384.1 | Unknown | | | |

# 5. DISCUSSION

## 5.1 Protein Candidate Selection through Bioinformatics Analysis

The purpose of this work was to explore the use of bioinformatics, together with protein array-based immunological screening, in discovering antigenic proteins for the serodiagnosis of *B. abortus* infection in cattle. While antigenic proteins have been deemed promising for the serodiagnosis of brucellosis across numerous *Brucella* species, the main serodiagnostic tool remains the use of the O-antigen to detect specific antibodies in sera of infected hosts. Identification of potential protein antigens for diagnostic applications has experimentally relied on proteomic techniques such as two-dimensional gel electrophoresis and mass spectrophotometry.

Few studies have incorporated bioinformatics tools into the discovery of novel diagnostic antigens for *B. abortus*. The present study demonstrated the successful application of bioinformatics tools to the identification of potential diagnostic protein antigens for *B. abortus*. The whole genome sequences of *B. abortus* strains available in the database of NCBI Microbial Genomes were analyzed with PSORTb. While thirteen strains were used for this study, a number of other strains' genomes were later fully sequenced, bringing the total number of complete genome sequences to nineteen. This offers the potential for a follow-up study searching for other protein candidates should the present experimental workflow prove to be feasible.

## 5.2 Success of *In Vivo* Cloning

The 100 candidate ORFs selected based on bioinformatics and literature review must be properly isolated and amplified in order to undergo *in vivo* cloning. Cloning of the candidates to appropriate vectors is critical to increase the copy number available for subsequent *in vitro* transcription/translation.

The linearization of the vector in preparation for cloning was achieved using digestion enzymes and confirmed with agarose gel electrophoresis. As seen in Figure 7, unsuccessful linearization is apparent by the smeared band. This band does not properly associate with the ladder in terms of its size because conformation affects the rate of migration(134). Super-coiled products run faster along the gel than linear fragments. Using the reference ladder for linear fragments, it is suggested that the circular product run is 2000 to 3000 bps. In reality, pIVEX2.3d used in the study is actually 3560 bps.

*Nde*I and *Not*IHF were preliminarily tested for use with pIVEX2.3d and pIVEX2.4d. Although the NEB protocol for each enzyme was followed, there was a clear difference in performance. In Figure 7, the *Not*IHF digestion had a faint secondary band below the target. Presence of secondary bands often indicates partial or incomplete digestion, as vectors can be in various degrees of the digestion when the process is terminated. A loosened but not completely uncoiled product has a tendency to run slower than the linear product(134). Adding further to the confusion, the band could also be contamination of some

sort. *Nde*I had a single band and was subsequently used for any vector digestion in order to prevent any downstream implications. Gel extraction of the linearized product further aids in the purification of the vector, eliminating any other discrete secondary products that could have been missed.

Colony PCR using primers for the T7 promoter and T7 terminator followed by agarose gel electrophoresis is capable of detecting the incorporation of the target into the vector through transformation and cloning. Vectors containing the ORF are significantly larger than an empty vector, with the change in size correlating to the length added from the T7 promoter and terminator, which equals to approximately 250 and 260 bp for either pIVEX2.3d or pIVEX2.4d. With the help of the DNA ladder, the change in size can be used to confirm the presence of the correct ORF. With the manipulations already performed to ensure homologous recombination occurs, such as the use of primers designed specifically for the ORF, the likelihood of improper incorporation and mis-orientation is low. Recircularization of an empty vector is comparable to the negative control measuring at 250 – 260 bp.

Overall, 91 out of the 95 candidate ORFs were successfully cloned. This success rate demonstrates that *in vivo* cloning combined with some *in vitro* manipulation makes the technique quite effective for high throughput application. The combination has resulted in a more robust and reliable cloning method. The successful application of this method to the cloning of *B. abortus* ORFs shows a promising cloning technique that is not commonly used but would greatly improve throughput and efficiency in the construction of recombinant plasmids.

## 5.3 Serum Dilution and Treatment for Antigenic Analysis

Serum is an important aspect of this stage of the project, as the determination of the candidates' antigenicity relies on their interactions with the antibodies in the serum. As such, the reactivity of the serum with antigens needed to be tested, with several sera provided by CFIA Ottawa Laboratory Fallowfield. An ELISA was required in order to determine the proper serum dilution required for testing. The concentration of antibodies present being too low would lead to weak signals and loss of antibody-antigen reaction amongst background signal for Western blots.

Problems can also arise with a serum containing a high concentration of antibody. High background noise can arise due to nonspecific binding (Precision Biosystems: Western Blot Troubleshooting Guide). As seen in Table 10, certain sera were too strong for the microplate photometer to read the OD values, indicating an incredibly high concentration of antibody. If used as a primary antibody in a Western blot, these sera would likely lead to false positive results as well as difficulty reading the blot due to saturation of the entire membrane.

Pre-adsorption is a method employed in order to increase the specificity of an antibody. By exposing the serum to other bacteria, non-specific antibodies bind to the bacteria and can then be removed, resulting in the elimination of these non-specific antibodies that would react with other non-

target proteins present in the recombinant antigen preparation during actual testing(126). When employing this method, *E. coli* is specifically used because of its known cross-reactivity in *B. abortus* tests as well as its involvement in the *in vitro* transcription/translation process through the *E. coli* HY kit(127). In theory, this should reduce the likelihood of selecting candidates that, in downstream applications, may lead to false positives.

The success of this method was tested with two sera of different reactivities: one moderately reactive and the other highly reactive. Pre-adsorption resulted in lower antibody-antigen interaction between the *E. coli* lysate negative control and the antibodies in the serum but did not eliminate it entirely, as seen in Figure 15 and Figure 16. This could be the result of incomplete purification by pre-adsorption, leaving non-specific antibodies in the serum, and would require optimization if this protocol were to be executed in a manner that would be applied to downstream applications. For the scope of this study, the addition of screening with pre-adsorbed serum is enough to show that cross-reactivity can occur and that this principle can be applied to reduce it in the future.

The binding of serum antibodies to LPS positive controls is where the difference in reactivity between sera becomes more prominent. S22, which is presumed to also have a lower concentration of antibodies when compared to the positive S+ serum, also shows a decrease in binding to the LPS control. The collective decrease in binding would indicate the success of pre-adsorption, decreasing the antibodies present in the serum. However, the serum with the higher concentration of antibody appeared to have equivalent binding to the LPS control when comparing pre-adsorbed and non-adsorbed serum. This can be explained by the higher prevalence of antibodies: despite the removal of cross-reactive antibodies, there were still sufficient excess *B. abortus* antibodies available for binding to the LPS antigen, saturating the control.

## 5.4 Expression of Recombinant Proteins by *In Vitro* Transcription/Translation

In order to test the reactivity of proteins encoded by the ORFs selected with the different sera, production of these proteins in a recombinant form becomes necessary. The desire for a speedy screening process has led to the choice of the *in vitro* transcription/translation strategy to produce recombinant proteins in a cell-free prokaryotic expression system, the RTS 100 E. coli HY kit.

To evaluate the kit for the production of recombinant proteins, a GFPuv gene cloned into the vector was used, together with the GFP plasmid supplied by the kit to serve as a positive control and with a negative control of water in the place of a vector. The success of the workflow (Appendix A - Workflow) was verified with the expression of GFPuv, which fluoresced alongside the control GFP (Figure 10). This success, which had not previously been achieved in the Lin lab, indicates that the entire process from PCR amplification to the *in vivo* cloning to the *in vitro* protein expression works well and has been optimized for the recombinant production of the protein candidates.

Upon demonstrating that the *in vitro* transcription/translation kit suited the needs of the study, it was tested with the 91 cloned ORFs. Detection of protein expression was accomplished by using Western blots with a penta-His mouse monoclonal antibody to target the C- or N-terminal His-tag in a recombinant protein. Initial cloning of the ORFs into the pIVEX2.3d vector resulted in the successful expression of 50 proteins. Expression of proteins from numerous cloned ORFs was not detected, indicating that some unknown factors may contribute to a failure in expression.

While the addition of a His-tag to a protein at the terminus is assumed to have no effect on protein, its location may alter the final structure, which may result in a negative effect on protein solubility(135). Should the His-tag be added to the terminal region that is critical to folding, the final protein structure may be misfolded and lead to altered functionality, including signalling or expression(136). The decision to use an N-terminal or C-terminal His-tag is still a consideration that is too frequently overlooked, considering there is experimental evidence that the addition of a tag can cause conformational changes to DNA-binding sites, particularly through the modification of disulfide-bonding patterns(137, 138).

A subset of the ORF candidates was selected on basis of size to be cloned into pIVEX2.4d, which expresses a protein with an N-terminal His-tag (Tables 6-10). Seventy ORFs were selected, including all those that had failed to express when cloned into pIVEX2.3d.

Within those seventy ORFs, a number of proteins that were effectively expressed when their corresponding ORFs were cloned into pIVEX2.3d were included in order for comparison. Of these, 23 were also expressed when cloning with pIVEX2.4d, but there were also several that did not. In the future, comparing how the structures differ between the addition of a C- or N-His-tag may be useful for downstream applications as the position of the His-tag is capable of influencing the folding(138) or affecting the protein expression. The addition of the His-tag was necessary for this study in order to screen for protein formation using the Western blot. Upon switching to pIVEX2.4d for cloning of the ORFs that fail to express protein in pIVEX2.3d, 17 more unique protein candidates were generated, bringing the total expressed proteins to 67, of the 100 original candidates.

## 5.5 Reactivity of Protein Candidates with Serum Antibodies

The use of protein arrays (dot blots) allows for the sera of different infected bovine to be tested for the reactivity against the protein candidates. Incubation of the proteins immobilized as an array on the membrane with a diluted serum allows the antibodies induced during infection to bind to any antigenic proteins.

The 67 unique recombinant protein candidates, for a total of 90 proteins expressed from the two different vectors, were first screened for the presence of a His-tag at the protein terminus. Doing this allows for the determination of relative concentrations of each candidate in order to assure that the future

assessments of antibody-antigen reactions are based solely on the strength of the interaction itself rather than the amount of protein present on the membrane. This method does its best to compensate for the difference in protein concentrations. Screening once again for the presence of a His-tag also acts as a secondary measure to ensure that the recombinant proteins have not degraded since their initial formation and testing, despite being stored at -20C.

The relative concentrations allowed for the adjustment of the volume being spotted onto the membrane for each candidate. With this configuration, the proteins were tested against the three serum conditions: S+, S22, and pre-adsorbed S22.

The initial screening included all 90 proteins that were detectable by anti-His-tag monoclonal antibody. The proposed method of determining antigenicity is based upon binding of the candidates with antibodies in the serum, which could be visualized through a secondary antibody. In order to present a more standardized method of comparison, protein-antibody interaction was calculated as the percentage of binding when compared relative to the LPS control, which was set as 100%. This gave some degree of quantitative analysis for the antibody-antigen interaction. This has been previously done, for example with *Mycobacterium tuberculosis*, calculating for a serum response intensity ratio against a control protein(139). In that study, only those sera giving a ratio greater than 1 were considered positive. In the present study, the LPS O-antigen generated such a strong signal response that no other protein candidates neared having a ratio of 1.

The pre-adsorption of a serum had the added element of decreasing background interaction and allowed for the proper analysis of serum antibodies binding to protein antigens, which had been reported previously in the literature(140, 141). The stronger antibody binding onto the LPS resulted in more intense dots, which is indicative of serum antibodies specific for *B. abortus* (Figure 15 and Figure 16). This mimicked a similar serum comparison Western blot assay for rickettsiosis(141), where pre-adsorption is a part of the standard procedure. This increase in binding to a control can skew the normalisation done referencing that control and subsequently drops the binding ratio of the protein candidates.

Ideally, the normalisation control would be one that is of medium intensity. However, LPS is one of the few universally accepted and well-studied antigens for *B. abortus*. As a result, no substitute for the control could be made and concessions had to be made. Instead of setting the threshold at a ratio of a protein candidate to the LPS control to be 1 (or 100%), the interactions of candidates to antibodies across the assay were assessed using the truncated average, as described in the materials and methods section. The exclusion of outliers when calculating the truncated average was an effort to counter the skewing from the potentially oversaturated and known strong antigen.

After this initial screening, the proteins to be screened were limited to those that were considered to be positive for the three serum conditions. Second and third screenings of those selected proteins were completed for biological reproducibility.

The initial results of the second and third rounds of protein array-based assays are largely similar to the findings of the initial test in that nearly all the reactive candidates remain positive in binding to serum antibodies. There were some candidates that demonstrated antigen-antibody interaction in two of the three tests. One such candidate, 19-11, had demonstrated significantly stronger binding in the screening process across all three serum conditions, which makes the absence of detectable binding during certain tests worth investigating in a follow-up study.

When re-examining the assays using the same relative binding method, the results became more interesting. Although twenty-four candidates had been selected based on their initial strong interaction with all three serum conditions, they did not all meet the desired 50% threshold (Table 12). Even so, some key candidates stood out. Candidates 11-05 and 21-05, which are in fact the same protein but cloned into pIVEX2.3d (11-05) and pIVEX2.4d (21-05) respectively, bound over the desired truncated threshold in all three tests for each condition. This same outcome was noted with another duplicate pair, 9-04 and 34-04. As a result, these two proteins should be in top consideration for further evaluation for diagnostic applications. In addition to these four recombinant forms of two protein candidates, three other candidates, 5-02, 14-11, and 33-01, demonstrated above-threshold binding to serum antibodies in all three rounds of protein array-based assays. Upon re-examining the assay results with the same 40% interaction threshold that allowed for the identification of additional candidates during the primary screening, five other candidates emerged with a strong possibility as antigenic proteins by meeting the new threshold in all nine test membranes (

Table 13).

Due to the novelty of this study, with the combination of techniques used, an arbitrary cut off was used. The average was used as a second measure to exclude any background interactions. However, by dropping the threshold to 40% of the truncated population, the positive binding of candidates per serum condition was more consistent across the three assays. As demonstrated by the shift in the inclusion factor, this threshold should be better examined both statistically and biologically through larger-scale validation studies in order to establish a practical threshold for the diagnosis. For example, it would be of interest to know how antibody response is induced during *B. abortus* infection, particularly those that target very specific antigens.

Overall, six of the proteins encoded by ten ORFs to be deemed antigenic were cytoplasmic proteins. Only two were on the outer membrane and none were extracellular. Further experimental work needs to be undertaken to determine if cytoplasmic proteins have a tendency towards antigenicity,

potentially through export to the extracellular space. The other two were of unknown localization. With future improvements on PSORTb or other predication tools, it would be interesting to see where those two candidates are ultimately located as well.

## 5.6 Antigenic Proteins

The two most promising antigenic proteins were WP_002963529.1 (a hypothetical protein) and WP_002965601.1 (4-hydroxy-tetrahydrodipicolinate reductase). Taking a closer look at the reductase, it is involved in amino acid biosynthesis as it partakes in lysine biosynthesis. In the past, reductases have been known to be antigenic. In fact, there is currently an FDA-approved diagnostic system for autoimmune diseases that targets a reductase(142).

# 6. CONCLUSION AND FUTURE DIRECTIONS

This study demonstrates promising protocols that can help identify the antigenic potential of proteins upon selection of candidates using bioinformatics. Ten candidates were determined to be antigenic but the most promising came from two specific proteins: WP_002963529.1 and WP_002965601.1. They were able to react with the antibodies in the sera when transformed and expressed using two different vectors.

In the future, evaluation of the newly identified antigens with more sera from infected and non-infected cattle is required in order to determine the accuracy of the new antigen-based tests as well as determine cross-reactivity. Further improvement of the pre-adsorption protocol for *B. abortus* serum will facilitate the discovery of novel antigens for the diagnosis of brucellosis. Alternatively, more candidates can be selected, expressed, and used to accelerate the diagnostic antigen discovery with the high throughput procedure established here. In addition, the determination of a practically meaningful threshold for specific antigen-antibody interaction using a large number of negative sera will be required in order to be able to label the proteins as being antigenic with a high degree of certainty.

This project was able to identify novel protein antigens, which can now be further explored and applied to downstream applications and demonstrated a procedure that can be applied to the search for antigenic proteins in other bacteria if so desired.

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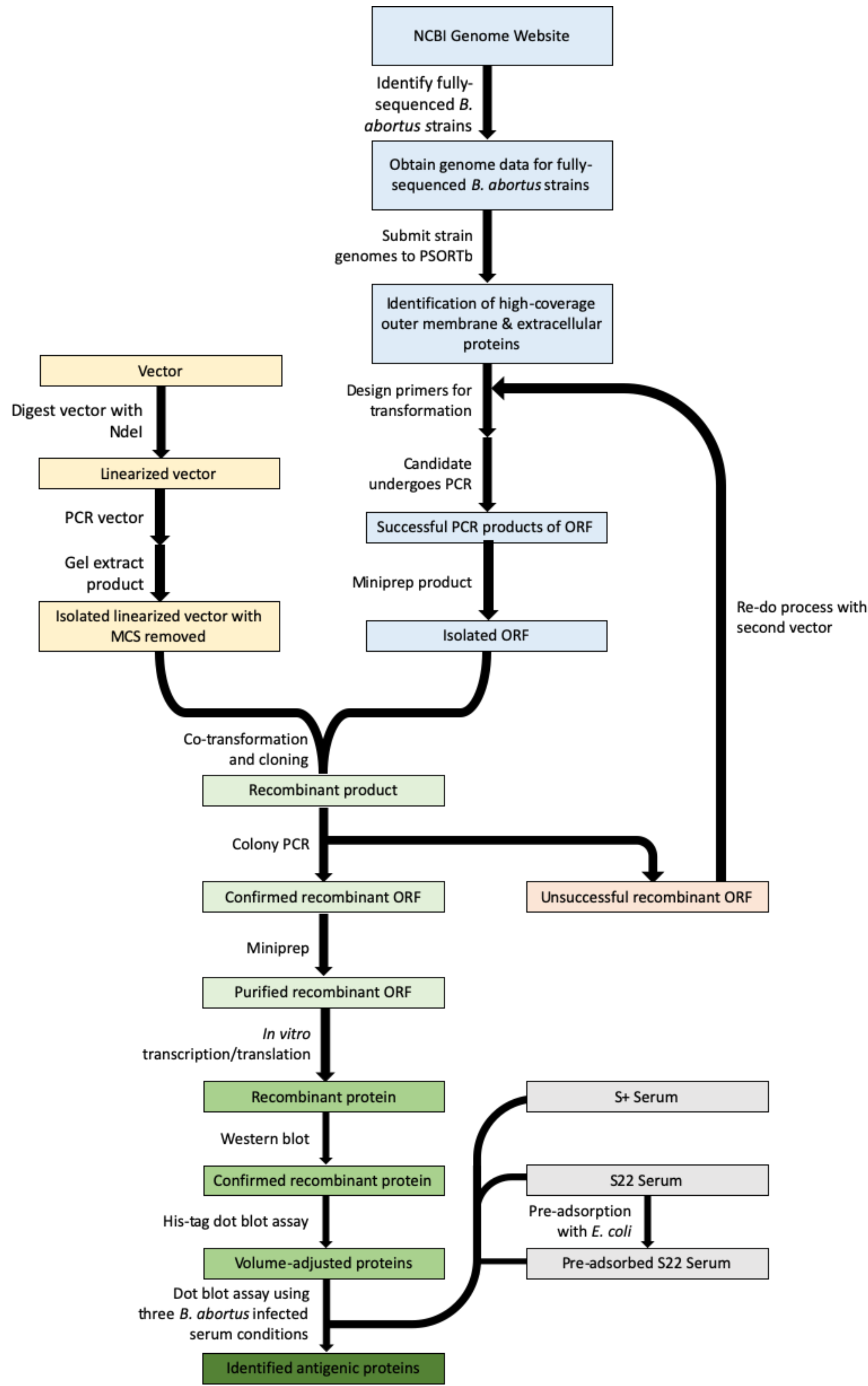
# 8. CONTRIBUTIONS OF COLLABORATORS

LPS-coated plates used in 3.6.1.1 as well as *B. abortus* sera were provided by CFIA Ottawa Fallowfield Laboratory.

## Appendix A - Workflow



## Appendix B – Common Buffers & Reagents

**Acrylamide/Bis (30%T, 2.67%C)**
For 500 mL:

| | |
|---|---|
| 146 g | Acrylamide |
| 4 g | N'N'-bis-methylene acrylamide |
| Up to 500 mL | $MQH_2O$ |

This must be done in a chemical hood.

**10% APS**
For 10 mL

| | |
|---|---|
| 1 g | Ammonium persulfate |
| Up to 10 mL | $MQH_2O$ |

Dispense into aliquots of 1 mL and sore at -20°C.

**3% BSA/1X PBS-TT**
For 1 L

| | |
|---|---|
| 30 g | BSA |
| Up to 1L | 1X PBS-TT |

Dispense into aliquots of 50 mL.
Store at -20°C.

**0.1M $CaCl_2$**
For 100 mL

| | |
|---|---|
| 1.47 g | $CaCl_2$ |
| Up to 100 mL | $MQH_2O$ |

Pass through 0.22µM filter and store at -20°C.

**0.1M $CaCl_2$ in 10% Glycerol**
For 10 mL

| | |
|---|---|
| 9 mL | 0.1M $CaCl_2$ |
| 1 mL | Sterile glycerol |

**Carbonate Buffer – 0.06M, pH 9.5**
For 1 L:

| | |
|---|---|
| 3.8 g | $NaHCO_3$ |
| 1.93g | $Na_2CO_3$ |

**Citrate Buffer – 0.05M, pH 4.5**
For 1 L

| | |
|---|---|
| 4.6 g | Citric Acid |
| 7.65 g | Tri-Sodium Citrate |

Store at 4°C.

**80 mM MgCl2 - 20 mM CaCl2**
For 100 mL

| | |
|---|---|
| 1.6264 g | Magnesium chloride |
| 0.2940 g | Calcium chloride |
| Up to 100 mL | $MQH_2O$ |

Pass through a 0.22 µM filter and store at -20°C.

**LB Agar**
Per liter:
40 g broth powder containing:

| | |
|---|---|
| 10 g | Tryptone Peptone |
| 5 g | Yeast Extract |
| 10 g | Sodium Chloride |
| 12 g | *Bacto Agar* |

Autoclave at 121 for 20 minutes.

If needed, add 100 ug/mL of carbenicillin.

**LB Buffer**
Per liter:
25 g powder containing:

| | |
|---|---|
| 10 g | Tryptone Peptone |
| 5 g | Yeast Extract |
| 10 g | Sodium Chloride |

Autoclave at 121 for 20 minutes.

If needed, add 100 ug/mL of carbenicillin.

**25x PBS**
For 1 L

| | |
|---|---|
| 27.5 g | $Na_2HPO_4$ |
| 7.88g | $NaH_2PO_4$ |
| 212.5g | NaCl |
| Up to 1 L | $MQH_2O$ |

Autoclave to sterilize.

**10X PBS-T – 0.1M, pH 7.2**
For 1 L

| | |
|---|---|
| 85.0 g | NaCl |
| 3.15 g | $NaH_2PO_4$ |
| 11.0 g | $Na_2HPO_4$ |
| 5 mL | Tween 20 |

Store at 4°C.

**PBS-TT**
For 10 L

| | |
|---|---|
| 400 mL | 25X PBS |
| 5 mL | Tween 20 |
| 20 mL | Triton X-100 |
| Up to 10 L | $MQH_2O$ |

Stir overnight.

**Protein Transfer Buffer**
For 1L:

| | |
|---|---|
| 5.82 g | Tris |
| 2.93 g | Glycine |

| | |
|---|---|
| Up to 1 L | MQ$H_2O$ |

Store at 4°C.

**10% SDS**

For 100 mL

| | |
|---|---|
| 10 g | SDS |
| Up to 100 mL | MQ$H_2O$ |

**2x SDS-PAGE Loading Buffer**

For 10 mL:

| | |
|---|---|
| 2 mL | 0.5 M Tris-HCl-pH 6.8 |
| 4 mL | Glycerol |
| 2 mL | Beta-mercaptoethanol |
| 0.4 g | SDS |
| 400 µL | 0.5% Bromophenol Blue |
| 1.6 mL | MQ$H_2O$ |

Dispense into aliquots of 1 mL and store at -20°C.

**10x SDS-PAGE Running Buffer**

For 1 L:

| | |
|---|---|
| 30 g | Tris |
| 144 g | Glycine |
| 10 g | SDS |
| Up to 1 L | MQ$H_2O$ |

**1X SDS-PAGE Running Buffer**

For 1 L

| | |
|---|---|
| 100 mL | 10X SDS-PAGE Running Buffer |
| 900 mL | MQ$H_2O$ |

**12% Separating Gel**

| | |
|---|---|
| 4.0 mL | Acrylamide/Bis (30%T, 2.67%C) |
| 2.5 mL | 1.5 M Tris-HCl-pH 8.8 |
| 3.35 mL | MQ$H_2O$ |
| 100 µL | 10% SDS |
| 50 µL | 10% APS |
| 5 µL | TEMED |

**SOC Medium**

For 100 mL

| | |
|---|---|
| 2 g | Tryptone |
| 0.5 g | Yeast Extract |
| 0.05 g | NaCl |
| Up to 100 mL | MQ$H_2O$ |

Sterilize by autoclaving and let it cool down to ~60°C.
Add 2 mL of 1 M Glucose.

**4% Stacking Gel**

| | |
|---|---|
| 0.65 mL | Acrylamide/Bis (30%T, 2.67%C) |
| 1.25 mL | 0.5 M Tris-HCl-pH 8.8 |

| | |
|---|---|
| 3.05 mL | MQ$H_2O$ |
| 50 µL | 10% SDS |
| 25 µL | 10% APS |
| 5 µL | TEMED |

**50x TAE**

For 1.5 L

| | |
|---|---|
| 363 g | Tris |
| 150 mL | 0.5 M EDTA pH 8.0 |
| 85.5 mL | $CH_3COOH$ |

**0.5 M Tris-HCl (pH 8.0)**

For 500 mL

| | |
|---|---|
| 30.275 g | Tris |
| Up to 500 mL | MQ$H_2O$ |

Adjust pH to 8.8 with 6 M HCl.

Store at 4°C.

**1.5 M Tris-HCl (pH 8.0)**

For 500 mL

| | |
|---|---|
| 90.8255 g | Tris |
| Up to 500 mL | MQ$H_2O$ |

Adjust pH to 8.8 with 6 M HCl.

Store at 4°C.

**ELISA Substrate Solution:**

| | |
|---|---|
| 0.5 mL | ABTS |
| 0.1 mL | H202 (3%) |
| 10 mL | Citrate buffer |

## Appendix C - Experimental Protocols

**Culture**

1. Streak *E. coli*-containing plasmids on a carbenicillin-LB plate.
2. Incubate at 37°C overnight.
3. Inoculate one colony into 5 mL of carbenicillin-LB broth, shaking at 200 rpm at 37°C overnight.
4. Spin down the cells at 13 000 rpm for 1 minute and discard supernatant.

**ELISA**

1. Remove pre-made plate from freezer and allow to thaw at room temperature for an hour.
2. Prime the ELISA Plate Washer, running the Quick program with $MQH_2O$.
3. When the program is complete, disconnect the tube and connect it to the wash buffer (PBST) bottle.
4. Repeat the program again, this time with the wash buffer, to prime the tube.
5. Once primed, the wash protocol is then run.
6. Prepare the serum.
7. Add 100µL of serum per well of the conjugate.
8. Incubate the plate for 1 hour at room temperature.
9. Prepare secondary antibody.
10. Wash plates with PBST.
11. Add 100µL of secondary antibody to each well.
12. Incubate the plate for 1 hour at room temperature.
13. Prepare ELISA substrate.
14. Wash plates with PBST.
15. Add substrate at 100µL per well and shake for 10 minutes.
16. Plate is then read by spectrophotometer.

**ELISA Plate Reading by Spectrophotometer**

1. Add color development substrate to blanking plate.
2. Load blanking plate (without lid or sealing film) and click “Read New”.
3. Save the data file.
4. Remove blanking plate from the carrier and load sample plate (without lid or sealing film).
5. Click “Read New”.
6. Open the data file and export the data file.

**iBind Flex Western Protocol**

1. Samples are dotted onto membrane at desired volumes.
2. Prepare 1X iBind Solution: 500µL iBind Flex 100X Additive + 10 mL iBind Flex 5X Buffer + 39.5 mL $MQH_2O$.
3. Immerse blotted membrane in 10 mL 1X iBind Solution.
4. Dilute each antibody as follows: 4mL 1X iBind Solution + 5x the manufacturer’s recommended dilution.
5. Place iBind Flex card on the iBind Flex device, aligned properly.
6. Apply 10 mL 1X iBind Solution to the card, saturating the Flow Region.
7. Add 1X iBind Solution to the Membrane Region.
8. Place the membrane on the card, protein side down, being sure to remove any air bubbles.
9. Close the iBind Flex device and add solution to the wells in the following order:
    a. Row 1: Primary antibody (4 mL)
    b. Row 2: 1X iBind Flex Solution (4 mL)
    c. Row 3: Secondary antibody (4 mL)

        d. Row 4: 1X iBind Flex Solution (12 mL)
10. Close the iBind Flex device well cover and leave the device to run for 2.5 hours.
11. Rinse the membrane in distilled water.
12. Perform HRP.

**_In vitro_ Transcription/Translation (RTS 100 _E. coli_ HY Kit)**

1. Prepare components by adding:
    a. 0.36 mL Reconstitution Buffer to *E. coli* lysate
        i. Aliquot 12 µL into 200 µL reaction tube
    b. 0.30 mL Reconstitution Buffer to Reaction Mix
    c. 0.36 mL Reconstitution Buffer to Amino Acids
    d. 0.33 mL Reconstitution Buffer to Methionine
2. Resuspend control by centrifuging down the Control Vector GFP and adding 50 µL of sterile water.
3. Set a water bath to 30°C.
4. Thaw reagents on ice.
5. Add the following into the reaction tube that already contains 12 µL *E. coli* lysate:
    a. 10 µL Reaction Mix
    b. 12 µL Amino Acids
    c. 1 µL Methionine
    d. 5 µL Reconstitution Buffer
    e. 10 µL of DNA Template (0.05µg/µL)
6. Incubate for 6 hours at 30°C.
7. Hold at 4°C overnight.
8. Analyze the product for expression by SDS-PAGE and Western blotting.
9. Aliquot 5 µL of the protein into tubes.

**Gel Extraction Protocol**

1. Prepare a 1% agarose gel, heating and mix solution until agarose powder has dissolved.
    a. 0.25 g of agarose in 25 mL of 1X TAE Buffer
2. Add 3.0 µL of SybrSafe (10 mg/mL).
3. When the solution has cooled, cast the gel with a 4 well comb (2 small wells, 2 larger wells).
4. Add 6X loading buffer to the sample and load the samples in each well.
5. Run for 80V for 50 minutes.
6. Under a UV light, visualize the fragment and excise it with a clean sharp scalpel.
7. Weigh the gel slice in a tared 2 mL tube. Max weight is 400 mg. If larger, divide in two.
8. Add buffer QG to the gel (3:1, 300 uL:100 mg).
9. Incubate at 50°C for 10 minutes and vortex every two minutes.
10. Add isopropanol (1 volume isopropanol:1 volume gel) and mix.
11. Apply the sample to the QIAquick spin column by adding 800 µL at a time doing the following between each addition:
    a. Centrifuge at 13 000 rpm for 1 minute.
    b. Discard flow-through.
12. Add 500 µL of Buffer QG to the column.
    a. Centrifuge at 13 000 rpm for 1 minute.
    b. Discard flow-through.
13. Add 750 µL of Buffer PE to the column.
    a. Centrifuge at 13 000 rpm for 1 minute.
    b. Discard flow-through.

14. Centrifuge at 13 000 rpm for another 1 minute.
15. Place column into a clean 1.5 mL tube and add 50 μL of Buffer EB, let it stand for 1 minute.
16. Centrifuge at 13 000 rpm for another 1 minute.
17. Spec the sample, using Buffer EB as the blank.
18. Store at -20°C.

**HRP Protocol**

1. Prepare substrate solution:
   a. 9 mL MQ$H_2O$
   b. 1 mL 10X HRP Color Development Buffer
   c. 2 mL Color Reagent A
   d. 60 μL Color Reagent B
2. Incubate the membrane on the shaker in the substrate solution at room temperature for 10 minutes.
3. Rinse the membrane with MQ$H_2O$ while shaking at room temperature for 5 minutes.
4. Change MQ$H_2O$ and shake for another 5 minutes.
5. Air dry the membrane overnight.

**Pre-adsorption of Serum**

1. Dilute serum in PBS-Tween 20 at desired dilution.
2. Serum is added to 1:10 dilution of *E. coli*.
3. Incubate at 37°C for 1 hour.
4. Microcentrifuge for 4 minutes.
5. Harvest supernatant.

**Preparation of $CaCl_2$ Competent *E. coli* Cells Protocol**

1. Streak *E. coli* from frozen stock on an LB plate (no antibiotic) and incubate at 37°C overnight.
2. Inoculate one colony into 5 mL of LB broth (no antibiotic) at 37°C overnight, shaking at 200 rpm.
3. Sub-culture at 1/100 dilution in 40 mL of LB broth.
4. Incubate while shaking at 37°C.
5. Measure OD every 20 minutes at 600 nm until a measurement of 0.35 is reached.
6. Transfer the culture to a sterile ice-cold 50 mL tube.
7. Cool down the culture on ice for 10 minutes.
8. Spin at 2700 x g at 4°C for 10 minutes.
9. Discard the supernatant and stand the tube in an inverted position for 1 minute.
10. Resuspend the pellet in 24 mL of cold 80mM $MgCl_2$ 20 mM $CaCl_2$ and rotate the 50 mL tube on ice for 20 minutes.
11. Spin at 2700 x g at 4°C for 10 minutes.
12. Discard the supernatant and stand the tube in an inverted position for 1 minute.
13. Resuspend the pellet in 1.6 mL of ice-cold 0.1M $CaCl_2$ in 10% glycerol and rotate in an ice tray on the belly dancer until completely resuspended.
14. Dispense the bacteria suspension in aliquots of 50 μL.
15. Store tubes at -80°C.

**Preparation of polyacrylamide gels for SDS-PAGE**

1. Assemble the gel cassette.
2. Prepare 12% separating gel:
   a. 4.0 mL Acrylamide/Bis (30%T, 2.67%C)
   b. 2.5 mL 1.5 M Tris-HCl-pH 8.8

    c. 3.35 mL MQ$H_2O$
    d. 100 μL 10% SDS
    e. 50 μL 10% APS
    f. 5 μL TEMED
3. Pour 3.5 mL into the gel cassette.
4. Add 100 μL of N-butanol to the top of the separating gel to level off.
5. Allow the gel to polymerize for 45 minutes to 1 hour.
6. Pour off the N-butanol and absorb remainder with a Kimwipe.
7. Rinse the gel surface 3x with MQ$H_2O$.
8. Prepare 4% stacking gel solution:
    a. 0.65 mL Acrylamide/Bis (30%T, 2.67%C)
    b. 1.25 mL 0.5 M Tris-HCl-pH 8.8
    c. 3.05 mL MQ$H_2O$
    d. 50 μL 10% SDS
    e. 25 μL 10% APS
    f. 5 μL TEMED
9. Pour the stacking gel on top of the separating gel.
10. Insert combs slowly.
11. Allow the gel to polymerize for 30 to 45 minutes.
12. Take out the casting frame and wrap it in Saran Wrap and store in a plastic bag at 4°C until use.

**Preparation of Protein Samples**

1. Boil water.
2. Add 15 μL of a protein sample to 14 μL of 2X SDS-PAGE Sample Buffer.
3. Mix well by vortex, lock the sample tube and place it on a floating tube rack in the boiling water for 10 minutes.
    a. Vortex every 2 minutes.
4. Let it cool down to room temperature.
5. Store at -20°C until use.

**QIA MINIPREP Protocol**

1. Resuspend cell pellets in 250 μL of Buffer P1.
2. Add 250 μL of Buffer P2 and gently invert the tube 4-6 times to mix (no vortexing).
3. Add 350 μL of Buffer N3, again gently inverting the tube 4-6 times.
4. Centrifuge at 13 000 rpm for 10 minutes at 4°C.
5. Taking the supernatant, apply it to the spin column in a tube using a pipette.
6. Centrifuge at 13 000 rpm for 1 minute.
7. Discard the flow-through.
8. Add 500 μL of Buffer PB to the spin column and centrifuge for 1 minute, discarding the flow-through.
9. Add 750 μL of Buffer PE to the spin column and centrifuge for 1 minute, discarding the flow-through.
10. Centrifuge again at 13 000 rpm for an additional 1 minute.
11. Place the spin column in a clean 1.5 mL tube.
12. Add 50 μL of Buffer EB to the spin column and let stand for 1 minute.
13. Centrifuge at 13 000 rpm for 1 minute.
14. Store the plasmid DNA at -20°C.

**SDS-PAGE Electrophoresis**

1. Assemble the gel cassette on the electrode stand and put it in the mini tank.
2. Fill the inner chamber with 1X SDS-PAGE Running Buffer.
3. Fill the lower chamber with 1X SDS-PAGE Running Buffer until the level is above the wire.
4. Load 10 μL of the SDS-PAGE sample and 5 μL of Pre-stained SDS-PAGE Standard Low Range.
5. Place the lid on the mini tank and run at 200V for 45 minutes.
6. The gel can then be processed for Western blot.

**Transformation of $CaCl_2$ Competent *E. coli* Cells by Heat Shock Protocol**

1. Set the dry bath at 42°C.
2. Remove appropriate number of vials of *E. coli* competent cells from -80°C and thaw on ice.
    a. Each vial contains 40 μL of competent cells.
3. Add 5-10 μL of ligation reaction (200 ng of insert and 100 ng of vector) or 2 μL of the positive control plasmid (0.5 ng/μL)
4. Mix with pipette tip (do not vortex).
5. Incubate on ice for 30 minutes.
6. Heat shock cells at 42°C for 30-45 seconds.
7. Hold the tubes on ice for 2 minutes.
8. Add 250 μL of room temperature SOC medium to each tube.
9. Incubate in the shaker at 37°C shaking at 300 rpm for 1 hour.
10. Plate entire transformation reaction on LB agar containing appropriate antibiotics.
    a. For positive control, plate 250 μL of 100-fold diluted transformation reaction
11. Incubate plates upside down at 37°C overnight.
12. Seal the plates with parafilm and store at 4°C.

**Western Blot**

1. Remove the polyacrylamide gel from the cassette.
2. Separate the stacking gel from the separating gel.
3. Soak the following in room-temperature Protein Transfer buffer:
    a. 2 thick blot papers
    b. 1 nitrocellulose membrane
    c. Separating gel
4. Assemble on the semi-dry transfer cell the following, in this order:
    a. Thick blot paper
    b. Nitrocellulose membrane
    c. Separating gel
    d. Thick blot paper
5. Between each layer, use a roller to remove air bubbles.
6. Put on lid and run the machine at 15V for 30 minutes.
7. On a shaker, incubate the membrane in 10 mL of 3% BSA/1X PBS-TT for 1 hour at room temperature.
8. Wash membrane with 10 mL of 1X PBS-TT for 3 minutes while shaking.
9. Incubate membrane while shaking with 10 mL of primary antibody solution at room temperature for an hour.
    a. Antibody solution: Monoclonal antibody hybridoma diluted 1:25 in 3% BSA/1X PBS-TT
10. Wash membrane while shaking 5 times with 10 mL of 1X PBS-TT for 3 minutes each.
11. Incubate the membrane while shaking with 10 mL of secondary antibody solution for an hour.
    a. Secondary antibody solution: HRP-conjugated goat anti-mouse IgG diluted 1:1000 in 3% BSA/1X PBS-TT

12. Wash membrane while shaking 5 times with 10 mL of 1X PBS-TT for 3 minutes each.
13. Perform HRP Colour Development.

Curriculum Vitae

**Teresa Nguyen**

**OBJECTIVE**
To obtain a position where I can maximize the use of and expand upon my research experience in order to contribute to the knowledge of the scientific community.

**CORE SKILLS**

Molecular biology
DNA and RNA extraction
PCR: conventional, qPCR, RT-qPCR
*In vivo* cloning and transformation
*In vitro* transcription/translation
Western Blotting
ELISA
Microbiology: *Listeria monocytogenes, Brucella abortus*
Cell culture
Passaging mammalian cell lines
Experienced in ISO 17025 labs

**WORK EXPERIENCE**

Sr. Research Technician July 2018 - Present
Spartan Bioscience

- Conducting day-to-day R&D experimental activities and following standard practices to obtain data
- Troubleshooting technical difficulties as required
- Developing genetic assays
- Extracting, integrating, compiling, and tabulating data

Laboratory Intern June 2015 – September 2015
ORISE

- Worked at U.S. Food and Drug Association laboratory on Dauphin Island, Alabama, via the ORISE Program
- Maintained cultured cell lines (HeLa, Vero, A549), including passaging and making the required media
- Experienced in conducting and optimizing RNA extractions, followed by working on them through RT-qPCR
- Concentrated and extracted enteric viruses such as norovirus and adenovirus to compare RT-PCR units vs. infectious units determined by plaque assay from the concentrate of the outbreak-associated shellfish
- Kept record of all results and protocol changes associated with sample variances
- Worked autonomously, planning and organizing project steps in advance in order to most efficiently book and utilize the lab equipment shared amongst the microbiology division.

Laboratory Intern September 2014 – December 2014
Bureau of Microbial Hazards, Health Canada

- Experienced in analyzing biological data from collected results that were organized in MS Word and Excel
- Isolated and cultured bacterial listeriosis pathogens on various types of media in order to facilitate the identification of the presence of a particular strain.
- Detected *L. monocytogenes* from food and clinical specimens
- Compared different protocols for rapid detection and confirmation of Listeriosis species
- Analyzed DNA of bacterial pathogens through extraction, polymerase chain reactions, and gel electrophoresis.
- Examined the usefulness of *Listeria* detection with novel loop-mediated isothermal amplification (LAMP) and compared it to mirrored qPCR.
- Followed good laboratory practice (GLP): experienced in aseptic techniques and respected ISO protocols and Standard Operating Procedures (SOP), echoing quality assurance and quality control (QA/QC) procedures

- Communicated and exchanged results to supervisors orally and written in reports
- Contributed in cross-country and cross-organization lab projects
- Participated in safe disposal of laboratory chemicals

**EDUCATION**
Master of Science, Candidate January 2016 - Present
**Microbiology and Immunology**
University of Ottawa

Baccalaureate in Science September 2010 – April 2015
**Honours Biomedical Science, Option in Cellular and Molecular Medicine (CO-OP)**
University of Ottawa

- Received University of Ottawa Admission Scholarship ($12 000)
- Graduated Cum Laude

**OTHER SKILLS**
Computer

- Microsoft Office
    - Learned independently to prepare and analyze data for written assignments and presentations with MS Word, PowerPoint, and Excel

Communication

- Spoken Languages: Fluent English, good French
- Written Skills:
    - Wrote English-language scientific reports for biology, chemistry, and biochemistry courses as well as co-op work terms
    - Wrote honours research project thesis
    - Drafted English meeting minutes for Omega Phi Sigma
- Oral Skills:
    - Headed weekly meetings
    - Completed honours seminar course
    - Presented honours research project at the University of Ottawa Poster Day
    - Presented recruitment campaigns for both Omega Phi Sigma and the Taler Lab

**Certificates**

- WHMIS
- Enhanced Reliability – Agriculture and Agri-Food Canada, Health Canada, CFIA
- TCPS2: Course on Research Ethics
- Ontario Hospital Association – Accessibility Customer Service Certificate