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In vitro and in vivo evaluation of a Brucella putative hemagglutinin

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IN VITRO AND *IN VIVO* EVALUATION OF A *BRUCELLA* PUTATIVE
HEMAGGLUTININ

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DEDICATION

For my family,

Leven & Beverly, Bernard & Jerry
Mike & Darlene, Nathaniel & Jennifer,
and David

Your contribution has been greater than you know.

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ABSTRACT

Brucellosis, a zoonotic disease caused by *Brucella* spp., presents both health and economic difficulties for livestock, wildlife, and humans. While brucellosis is nearly eradicated in the United States, the disease remains detrimental in many countries worldwide.

Attempts to produce a safe and effective small ruminant vaccine have been met with limited success. The current vaccine for bovine brucellosis in the United States is *B. abortus* RB51. This strain transiently colonizes the host and induces a cell-mediated immune response. Levels of protection have not been demonstrated in goats and thus it is considered a relatively poor caprine vaccine that probably does not survive long enough in the tissues to produce a sufficient protective immune response.

This study analyzes the possibility of using RB51 containing plasmid QAE for vaccination in goats. The plasmid QAE contains a gene region of DNA from *B. melitensis* 16M hypothesized to encode for a putative hemagglutinin that is believed to be a host specificity protein. The region E gene sequence is not present in any strain of *B. abortus*, including RB51. It is proposed that the addition of region E to RB51 will enhance survivability in the caprine host to the extent where an adequate protective immune response is elicited.

The QAE plasmid was electroporated into *B. abortus* RB51 and screened using ampicillin resistance as a selective measure. Ten goats were conjunctivally inoculated with RB51-QAE, the modified strain, and ten goats received strain RB51 in the same manner as controls. Two goats from each group were euthanized and necropsied at weekly intervals for four weeks and again at 42 days. Tissues from these animals were

taken at necropsy and used to collect data for colonization and histopathology. Serum samples were also analyzed for *Brucella*-specific antibodies. Both strains transiently colonized the hosts without producing any detrimental pathology. However, the RB51-QAE goats demonstrated higher levels of colonization and greater humoral immune responses for longer periods of time. These are very promising findings as the levels of colonization and humoral responses may correspond with better protection. These results warrant further testing of RB51-QAE as a potential vaccine for caprine brucellosis.

The putative hemagglutinin was characterized using hemagglutination assays, absorption assays, and mass spectrometry analysis following 2D gel electrophoresis. The region E protein was found to provide RB51-QAE with increased hemagglutinating and immunogenic capabilities. Furthermore, there is evidence to suggest that the protein is a cell surface protein, not excreted from the cell.

The accepted gold standard for the detection of brucellae is bacterial culture. PCR is being evaluated as a possible alternative detection method for *Brucella* spp. in bodily fluids such as blood and urine. Currently, there is no accepted standard for detection of brucellae using PCR.

The effects of template preparation, primer selection, and PCR optimization on the limit of detection for *B. abortus* 2308 and *B. melitensis* 16M in association with whole blood, plasma, or urine were examined. Ten-fold dilutions were made from a known number of bacterial cells in each of the fluids tested. The practices of using whole killed cells as a direct template for PCR as well as two differing DNA isolation techniques were evaluated for each fluid dilution. Our findings suggest that a more extensive template preparation technique and PCR protocol elongation can greatly

improve the limit of detection capabilities. Biological fluids provided dissimilar results based on the PCR inhibition properties of the fluid and DNA isolation techniques. The results of this experiment encourage further investigation into the optimization of conventional PCR techniques as a faster and more efficient diagnostic tool for *Brucella* spp. in humans and animals.

INTRODUCTION

Brucellae are aerobic, Gram-negative, facultative intracellular pathogens that are the causative agents of brucellosis in both humans and animals. *Brucella abortus* is responsible for bovine brucellosis and in other primary hosts such as moose, elk, and bison. Secondary hosts of *B. abortus* include goats, sheep, pigs, and humans. *Brucella melitensis* is the most virulent *Brucella* spp. and infects mainly goats, although humans are a secondary host. Other species of *Brucella* include *B. suis*, *B. ovis*, and *B. canis*. These species infect pigs, sheep, and dogs, respectively, as primary hosts. A recently proposed species, *B. microti*, infects voles and foxes and is genetically homologous to other species of brucellae. There are also two species, *B. ceti* and *B. pinnipedialis*, found to infect marine mammals as primary hosts and current research suggests that both are zoonotic pathogens (Foster et al. 2007). Three species, *B. melitensis*, *B. abortus*, and *B. suis*, are considered agents of bioterrorism by the United States Centers for Disease Control and Prevention because they are easily disseminated, highly contagious, and stable under conditions of production and storage. Widespread disease would pose a threat to public health for humans and companion animals, and have devastating effects on the agricultural industry.

Transmission among animals is most often through direct contact of contaminated substances with the mucous membranes. Human infection occurs through direct contact with infected animals and the consumption of their unpasterurized dairy products. The disease is not transmitted from human to human. Other modes of transmission can occur through contact with abraded skin and aerosolization. Once in a susceptible host, brucellae eventually disseminate throughout the host, colonizing tissues and affecting

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several systems detrimentally. Human brucellosis is primarily characterized by undulating fever. Animal brucellosis is characterized by abortions and other pathologies of the reproductive organs, as well as joint abnormalities.

Standard diagnosis of *Brucella* infection is performed by blood testing and culture. Serologic diagnosis is not foolproof and can provide false positive results due to antibodies produced by smooth vaccine strains or other bacteria with homologous antigenic determinants. Bacterial culture is a long process and brucellosis may not be correctly diagnosed for several days to weeks following sample collection. Furthermore, a faster, more efficient, and reliable diagnostic method would improve the detection and treatment capabilities of brucellosis in both humans and animals.

Complete genomic sequences have been published for several species of *Brucella*, which have been essential in the discovery and characterization of virulence factors employed by the organism under both *in vitro* and *in vivo* conditions. This study focuses on the characterization of a putative hemagglutinin gene, named region E, located on the chromosome of *B. melitensis* 16M that is absent in all strains of *B. abortus* (Perry 2007). Protein characterization was performed under both *in vitro* conditions and *in vivo* in the caprine model comparing a vaccine strain of *B. abortus* RB51 to a strain containing the putative hemagglutinin gene expressed *in trans*, *B. abortus* RB51-QAE. These experiments were carried out for the purpose of characterizing the region E putative hemagglutinin and evaluating its potential role in the development of a more effective rough vaccine. Rough vaccines are not highly effectual in small ruminants and the development of a more efficacious vaccine would contribute tremendously to the eradication efforts in endemic countries.

PCR has been explored as an improved technique for the diagnosis of brucellosis in humans and animals. There is currently no standardized technique for brucellae and findings have been mixed. This study explored the limit of detection of *Brucella* cells by conventional PCR in several easily-attainable bodily fluids to explore the possibility of using PCR as a standard diagnostic tool for brucellosis.

LITERATURE REVIEW

Genus Brucella

Bacteria in the genus *Brucella* are the causative agents of brucellosis in humans and animals. *Brucella* spp. are important zoonotic and agricultural pathogens that infect a wide range of hosts with varying degrees of pathology. Although rare in the United States, brucellosis in both humans and animals remains a threat to public health and agricultural economy in endemic areas.

Human brucellosis is uncommon in the United States with an estimated 100 cases annually. The disease poses more serious problems in underdeveloped countries around the world (Sauret et al. 2002, Seleem et al. 2009). Affected regions worldwide include Latin America, southern Europe, the Middle East, areas in Asia and Africa, and countries of the former Soviet Union. Classical species of *Brucella* that are infectious to humans include *B. melitensis* (biovars 1-3), *B. abortus* (biovars 1-6, 9), and *B.suis* (biovars 1,3,4). *B. canis* infections occur in immunocompromised individuals, but only rarely. There is no approved vaccine for protection in humans and vaccines approved for use in animals are infectious to humans. Transmission is most commonly through ingestion of unpasteruized milk from an infected animal, direct contact with infected birth materials, or during slaughter. Infection of humans is also possible through the aerosol route (Heymann 2004, Smither et al. 2009). Although a reportable disease in most countries, it is believed that the actual number of reported cases worldwide represents only a portion of the actual cases (Sauret et al. 2002, Seleem et al. 2009).

Brucellosis is nearly eradicated in domestic animals in the United States through a system of successful vaccination regimes and diagnostic testing procedures. Currently,

domestic livestock in the United States are routinely tested for brucellosis using standardized diagnostic procedures, such as the Rose Bengal and the Plate Agglutination test, which are used to screen domestic herds (Robinson 2003). Those animals found to be brucellosis suspects are subjected to further testing, and if confirmed positive, are slaughtered for removal from the herd population (USDA 2006). Additional serological tests are then conducted periodically following the removal of brucellosis seropositive animals until the herd is considered completely negative (Ragan 2002). Prevention by vaccination is a key tool in the eradication of brucellosis through preventative measures and is implemented in domestic herds in the United States (USDA 2006). The current vaccine used in the United States is the *B. abortus* RB51 rough vaccine, which replaced the *B. abortus* Strain 19 (S19) smooth vaccine in 1996 following complications in diagnostic testing with S19 (USDA 1996).

Continued herd management is crucial in the eradication program. Herd management procedures assess the risk of a herd becoming exposed to and acquiring brucellosis, implementing measures to reduce that risk, and perform routine testing to ensure early detection (USDA 2008). Domestic livestock, especially those herds located in areas that are high-risk for exposure, are routinely tested and closely monitored for any signs of brucellosis and the occurrence of any abortions are thoroughly investigated. Herds found to include brucellosis positive animals are quarantined until further testing clears the herd of any suspect infections. Furthermore, any additions to a herd are tested and confirmed brucellosis negative before entering the population (USDA 2006). The control measures of this eradication program have proven successful in eliminating the disease from domestic livestock in the United States (USDA 2008).

Because it is more difficult to test wild animals, the status of brucellosis in wildlife is not fully understood. Specifically, free-ranging elk and bison in the Greater Yellowstone Area (GYA) pose the greatest obstacle to complete eradication in the United States. Feed grounds have been developed where grazing herds of possibly infected wild and domestic animals frequently congregate and intermingle. Because brucellosis is spread primarily through contact with the fluid secretions, such as mucus, milk, maternal and fetal tissues and other byproducts of abortion, one infected animal can quickly infect several grazing herds through contact or exposure at a single feeding ground. Studies have shown that fluids from infected animals that are deposited on the ground can remain infective to other animals for periods of over 100 days, given the sample is in a cold, shaded environment (Timoney et al. 1988).

B. melitensis, *B. abortus*, and *B. suis* are designated as class B, biosafety level 3 select agents by the Centers for Disease Control and Prevention. These species are infectious to humans and are easily disseminated for potential weaponization (Pappas et al. 2006). Brucellosis as a bioterrorist agent has the potential to cause devastating consequences for agricultural and public health systems.

Brucella spp. are Gram-negative, non-motile, non-encapsulated coccobacilli that are the causative agents of brucellosis in animals and humans (Corbel 1997). Most, but not all, species express a complete lipopolysaccharide (LPS) outer membrane component. The LPS provides protection from certain host defenses and structural support. The outermost fraction of the LPS is the O-polysaccharide group (OPS) which compositionally unique in *Brucella spp*. and highly antigenic. Species of Brucella that express a functional OPS group are designated as "smooth", whereas species that do not

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express a functional OPS group are designated "rough". Ideal laboratory growth of brucellae is performed with selective, nutrient-enriched media in an aerobic 5% CO_2 atmosphere at 37°C over a period of 48-72 hours. Most classical species of brucellae provide a positive reaction for catalase, oxidase, and urease tests, with the exception of *B. ovis*, which is oxidase and urease negative, and *B. neotomae*, which is oxidase negative (Yagupsky et al. 2005).

Pathology

Once an animal or individual becomes infected with the *Brucella* pathogen, the bacterium travel in the lymph to the nearest draining lymph node and then into the blood stream of the host animal. *Brucella* spp. avoid destruction by the host's innate immune system with an ability to avoid lysosomal degradation after being engulfed by host macrophages (Celli 2006). Furthermore, *Brucella* spp. thrive and proliferate inside of the host macrophage, and ultimately cause the death of that macrophage, releasing the multiplied bacteria back into host tissues. Once the host becomes bacteremic, the pathogen migrates throughout the body to different organs and tissues (Cheers et al. 1984).

The most common pathology of brucellosis in animals is the abortion of the first pregnancy that occurs after the onset of the disease. Most *Brucella* spp. proliferate efficiently in the cells of the body with high erythritol levels, such as the cells found in the urinary and reproductive tracts (Enright 1990). Once in the uterus, the bacteria can penetrate the epithelial cells of the embryonic membrane and cause placentitis, which can lead to a decrease in the blood supply to the fetus. Fetuses recovered from brucella infected mothers have also been shown to develop edema and congestion in their lungs

and hemorrhages in their epicardium. Whether the cause is a decrease in oxygenated blood due to an inflamed placenta or the detrimental lesions formed by the infection of the fetus, the final outcome of pregnancy is either an abortion or a weak fetus that dies shortly after birth (Enright 1990).

Other effects on the reproductive system of animals with brucellosis include the swelling of the testes and epididymus of male animals. As in the uterus, *Brucella* in the male urogenital tract can also cause inflammation and swelling of the male reproductive organs. Orchitis and epididymitis are common symptoms of brucellosis in animals, causing sterility in infected males. Most, if not all, *Brucella* species can be isolated from semen, and males can shed the bacterium for prolonged periods (Iowa 2009). Brucellosis is also responsible for arthritis of the joints and spondylitis in many infected animals as well as hygroma formation on the knees. Brucellosis can also be responsible for mastitis in females, neurologic swelling and splenic abscesses with edema, congestion, and hemorrhage in the lung and heart.

Granulomatous inflammation is also observed during persistent *Brucella* infections. The exudate of granulomatous inflammation is generally composed of mature macrophages, which gravitate to the area of the body affected by the causative agent (McLaughlin 2007). Here, macrophages have the potential to form into multinucleate giant cells. Multinucleate giant cells are a hallmark of infections involving pathogens that thrive within phagocytes, as infected macrophages are identified and engulfed by other macrophages in a process that results in the formation of a giant cell composed of several responding phagocytes (McLaughlin 2007, Abbas 2005). Fibrosis and necrosis are often associated with granulomatous inflammation, especially in the case of

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granuloma formation. Granuloma formation is primarily a product of chronic delayed-type hypersensitivity and occurs in cases of chronic brucellosis. In a persistent infection and subsequent immune response, the lytic and anti-pathogenic actions of non-specific phagocytes begin to damage host tissue. In a chronic infection, this is sometimes manifested as the formation of fibrosis and scar tissue surrounding the site of inflammation, which has the potential to cause tissue damage that may disrupt normal tissue function (Merck 2007). Necrosis formation in the center of granulomas may occur due to anoxic conditions and may function to eliminate infected macrophages, destroying the pathogen within (Abbas 2005).

Species of Brucella

Brucella melitensis

Originally named "*Micrococcus melitensis*", *Brucella melitensis* was discovered by Lieutenant Colonel David Bruce in 1887 from British soldiers residing in Malta. *Brucella melitensis* is a world-wide disease which remains most problematic in developing countries. Although considered to be eradicated in the United States, *B. melitensis* is a continuing cause for concern because of its status as a potential agent of biological warfare (Moreno et al. 2002).

The primary host of *B. melitensis* is the caprine host as well as the ovine host, although the pathogen can be problematic in cattle, camels, and humans as secondary hosts (Nielson et al. 1990). In female goats and sheep the primary symptom is abortion, but larger doses of the pathogen cause mastitis and joint problems. In male goats, orchitis is uncommon, and if observed it is generally unilateral. In male sheep, however,

the disease is often asymptomatic, although orchitis and hygromas are a rare observation (Alton 1990).

The only approved vaccine against *B. melitensis* is the Rev.1 vaccine, developed from a laboratory strain of *B. melitensis* 16M. This vaccine, although effective, is potentially abortigenic and produces anti-LPS antibodies that can interfere with diagnostic tests (Gonzalez et al. 2008, Blasco 2006). Vaccines developed from strains of *B. abortus* are not effective in providing protection against infection with *B. melitensis*.

B. melitensis is the most virulent strain in human infection, with only 1-10 organisms needed for infection and with undulant fever as the primary symptom (Mantur et al. 2007). There is no human vaccine for brucellosis and the *B. melitensis* Rev.1 vaccine for animals is infectious to humans (Seleem et al. 2009). *Brucella melitensis* is highly contagious and can be easily disseminated with devastating effects to public and agricultural health. *B. melitensis*, as well as *B. suis* and *B. abortus*, have been deemed potential bioterrorism agents by the United States Centers for Disease Control and Prevention.

Brucella abortus

Brucella abortus is the agent responsible for the majority of bovine brucellosis cases. It was discovered in 1897 by Bernhard Bang and the disease was originally called "Bang's disease" (Mochman et al. 1988). In 1918 Alice Evans linked *B. abortus* with the previously discovered *B. melitensis* and showed that both were agents of brucellosis in humans and animals (Parascandola 1998). *Brucella abortus* is distributed worldwide and is still somewhat problematic in the United States, with wildlife remaining the most prevalent reservoir.

Primary hosts include cattle, bison and elk, but goats, sheep, pigs, and humans are also susceptible as secondary hosts. Affected cattle typically experience a permanent infection, shedding the bacterium for the duration of their lives (Nielson et al. 1990). The disease is spread easily through direct contact and an entire herd can quickly become compromised with the inclusion of a single infected animal. Bovine brucellosis is a disease that primarily targets the reproductive system of cattle (Enright 1990). Infected cows typically experience joint ailments, a decrease in milk production, sterility, and abortion of the first pregnancy that occurs after the onset of infection (Nielson et al. 1990). In bulls, orchitis can develop. Although rare in the United States in domestic cattle today, *B. abortus* remains a threat to wildlife species such as moose, elk, caribou, and bison.

Brucella abortus in wildlife, particularly in the Greater Yellowstone area (GYA), is difficult to control and monitor. The presence of brucellosis in the GYA was first described in 1917 and it has persisted in Yellowstone bison and elk herds ever since. Domestic cattle in and around the GYA may contract brucellosis from these reservoirs (Davis and Elzer 2002). For this reason, the prevalence of brucellosis in free-ranging wildlife of the GYA has proven to be a major hindrance in efforts to eradicate the disease completely in the United States. Management practices implemented for the eradication of brucellosis in domestic herds are not as easily applied to wildlife. Diagnostic testing and removal of infected animals are difficult due to the relative inaccessibility of animals and migration through multiple management jurisdictions (Etter and Drew 2006). Vaccines developed for use in domestic livestock have shown a lesser effectiveness in wildlife and present difficulties in terms of vaccine delivery (Davis and Elzer 2002).

Vaccination against *B. abortus* has proven an important tool in eradication of the disease. The first brucellosis vaccine approved for commercial use was labeled Strain 19 (S19) and was derived from a field strain of *B. abortus* (Sanmartino 2005). However, S19 is a smooth organism and induces the production of antibodies that interfere with diagnostic tests. For this reason, a rough *B. abortus* vaccine, termed RB51, was developed from a laboratory strain of *B. abortus* 2308 and was shown to be equally efficacious as S19 and did not hinder serological testing (Schurig 2002, 1991). RB51 remains the only approved vaccine against *B. abortus* in the United States.

Humans, although not a primary host, can contract brucellosis through contact with infected animals. *Brucella abortus* is moderately infectious to humans, with an estimated 1×10^5 organisms needed for infection and the primary symptom is undulant fever. While *B. melitensis* is a more frequent and wide-spread zoonosis, *B. abortus* infects mainly occupational groups working closely with the organism or infected animals (Seleem et al. 2009). *Brucella abortus* vaccines approved for use in animals, such as S19 and RB51, are infectious to humans and there remains no vaccine available for human use against *B. abortus*. The largest economic and sociological threat from *B. abortus* is to that of the cattle industry, where it is capable of severe economic damage.

Brucella suis

*S*wine brucellosis was first reported in 1914 in a government report by Traum (Traum 1914) and was assumed to be caused by a strain of *B. abortus*. It was not until 1929 that the disease pathogen was recognized as closely-related, yet separate from *B. abortus* and renamed *B. suis*, denoting the primary host (Huddleson, 1929). *Brucella suis* maintains a serious presence in feral swine and has not been eradicated from domestic

swine in the United States. All states, with the exception of Texas, maintained a swine-brucellosis-free status as of 2008 (USDA 2008), but Florida and Hawaii remain problematic areas.   The intermingling of domestic swine with a largely infected feral swine population poses an obstacle in eradication of the disease from commercial swine herds.

Brucella suis is most often described in pigs, but reindeer are also a primary host for B. suis biovar 4. Secondary hosts include cattle, horses, dogs, and humans. Transmission is most often accomplished through direct contact with the reproductive byproducts of infected sows. An additional aspect of B. suis transmission is that the organism can also be transmitted venereally from infected boars (Alton 1990). The disease in swine is similar pathogenically to brucellosis in other animals. The most common symptom is the incidence of abortions in pregnant females, but other symptoms unrelated to the reproductive organs may occur, such as pain in the joints and spondylitis (Conger et al. 1999). Brucella suis often presents a prolonged bacteremic phase in swine which can last up to 34 weeks (Alton 1990). Brucella infected herds of reindeer have been recorded across the northernmost regions of North America, with the majority of infections occurring in the Arctic Circle (Zarnke 2001, Tessaro et al. 1986). There are currently no vaccines derived from any B. suis strain and no commercially available vaccine against B. suis infection for humans or animals in the United States. Studies focused on the development of an effective B. suis vaccine are ongoing.

B. suis is moderately to highly infectious to humans, requiring contact with approximately 1×10^3-1×10^4 organisms for infection. Because of the lengthy bacteremic phase seen in swine, people working with infected pigs are at a higher risk of contracting

brucellosis than those working with other animals (Conger et al. 1999). The highest risk

for contracting brucellosis through *B. suis* is seen in people working in close proximity

with the pathogen or infected animals. Undulant fever remains the primary symptom in

individuals infected with *B. suis*. This pathogen was weaponized via the M33 cluster

bomb by the United States in 1955 and all known US stocks were destroyed by the mid-

1990s (Smart 2008, Croddy et al. 2005).

Brucella ovis

Brucella ovis was originally described in New Zealand and Australia by

McFarland et al. and Simmons et al., respectively (Blasco 1990). *Brucella ovis* is most

prevalent in areas associated with sheep farming and cases have been recorded in New

Zealand, Australia, North and South America, South Africa, and several European

countries (Iowa 2009). *Brucella ovis* differs from the previously discussed species in that

it is a naturally rough strain that is infectious even without an intact lipopolysaccharide

(LPS), which lacks the O-polysaccharide side chain (OPS) (Buddle 1956). Other

differences are noted in biochemical tests in that *B. ovis* is found to be oxidase and urease

negative, whereas *B. melitensis*, *B. abortus*, and *B. suis* are positive for both.

Brucella ovis primarily infects rams while ewes and goats serve as other hosts for

the organism. This pathogen has also been documented to infect farmed red deer in New

Zealand. Transmission is often venereal and ewes carry the pathogen vaginally for two

months or more and shed the bacterium in their vaginal discharges and milk. Infected

rams have been shown to shed the bacterium in semen for up to 4 years or longer and

shedding in the urine has also been documented (Iowa 2009, Cerri et al. 1999). As with

other previously discussed species, direct contact with infected mucus membranes is

another route of transmission. The incubation period in rams is as long as 3-8 weeks

post-inoculation. Symptoms of *B. ovis* in rams include orchitis, epididymitis, and

impaired fertility or sterility. Ewes are less likely to show symptoms if infected, but

infrequent abortions and placentitis can occur (Iowa 2009, Grillo et al. 1999).

There are no vaccine regimens practiced for protection of rams against *B. ovis* in

the United States. New Zealand employs a commercially available vaccine and most

applicable countries vaccinate young rams with *B. melitensis* Rev.1 (Iowa 2009, Jimenez

et al. 1994). Preliminary studies using mutant forms of the Rev.1 vaccine in sheep for

protection to *B. ovis* challenge infection have been documented (Grillo et al. 2008). The

test and slaughter method is used commonly to rid herds of infection. Antibiotic

treatment has been attempted in valuable rams but generally is not feasible for treatment

of a herd. *B. ovis* is not infectious to humans and although potentially detrimental to the

sheep industry, not considered a bioterrorist threat.

Brucella canis

Brucella canis was first described by Carmichael and Bruner in 1968 and is the

cause of brucellosis in dogs and other canids. The disease became of interest after a large

number of abortions were noted in beagles in 1966 (Morisette 1969). Geographically,

cases of *B. canis* have been documented in the United States (particularly the southern

states), Mexico, Canada, Central and South America, some European countries, Tunisia,

Nigeria, Madagascar, Malaysia, India, Korea, Japan and China. *Brucella canis*, like *B.

ovis,* is a species of *Brucella* that has a naturally rough phenotype in its infectious form.

The primary host for *B. canis* is the canine, and both male and female animals are

affected. Transmission of the organism can be venereal or through contact of mucous

membranes with urine or with infectious tissues following an abortion. Females have been shown to shed the bacterium in vaginal discharges for up to 6 weeks following an abortion. Males have been shown to shed the bacteria sporadically for years after initial infection in semen and urine. Live *B. canis* can also be found in milk, urine, saliva, nasal and ocular secretions, and feces of infected animals (Ledbetter et al. 2009, Wanke et al. 2004). Canine brucellosis is typically manifested as late-term abortions in females and sterility in males. Seemingly healthy pups may be born congenitally infected and develop symptoms later in life (Iowa 2009). In males, orchitis, epididymitis, scrotal edema, and sterility have been observed. Other symptoms may include lymphadenitis, fatigue, loss of appetite, loss of alertness, and behavioral abnormalities. Infected animals often do not appear outwardly ill but can remain infected for up to 5 years (Wanke et al. 2004). Canine brucellosis is rarely fatal. Antibiotic treatment is available, although some forms of treatment are expensive while others are not always effectual. The greatest economic impact of the disease is seen in breeding kennels, where up to 75% fewer puppies have been noted in some affected kennels (Iowa 2009). There is no vaccine available for *B. canis,* and control is most often performed by removal of infected animals and sanitation of the kennel or living space previously occupied by the animal.

Human infection is rare and normally occurs in immunosuppressed individuals if contact of the mucosal membrane or abraded skin occurs with $>1\times10^6$ organisms. Symptoms in people infected with *B. canis* are similar to those seen with other species of zoonotic *Brucella*. Although it is a zoonotic pathogen, *B. canis* is not considered to be a potential agent of bioterrorism.

Brucella neotomae and Other Proposed Brucella Species of Terrestrial Animals

Brucella neotomae was first described in 1957 after isolation from the desert wood rat in the United States by Stoenner and Lackman (Cameron and Meyer 1958). *Brucella neotomae* is a smooth organism, has a distinctive metabolic pattern, and performs similarly on biochemical tests used to identify other species of *Brucella* (Meyer 1990).

Most recently, *B. microti* has been described as a *Brucella* species isolated from common voles in the Czech Republic and red foxes in lower Austria (Scholz et al. 2009). Another proposed species of Brucella is the newly described *B. inopinata,* which was isolated in Germany from a breast implant wound of a 71-year old woman. *B. inopinata* is genetically highly homologous to other recognized *Brucella* strains but exhibits a unique 16S gene sequence (Scholz et al. 2009). Studies on these new proposed strains are ongoing. Further research is needed to determine their threat to agriculture or economy.

Brucella ceti and Brucella pinnipedialis

Brucella ceti and *B. pinnipedialis* have been recently described as the cause of brucellosis in marine mammals (Foster et al. 2007). In 1990, a common dolphin from the English coast tested seropositive for anti-*Brucella* antibodies, sparking research into the field of marine mammal brucellosis worldwide (Jepson et al. 1997). These two species were placed in the *Brucella* genus because they are aerobic, non-motile and catalase-positive. Furthermore, these species have a greater than 77% homology to other species in the genus *Brucella* (Foster et al. 2007). *Brucella ceti* is the name given to the novel *Brucella* species causing brucellosis in cetaceans (such as whales, dolphins, and

porpoises). *Brucella pinnipedialis* is the name given to the novel *Brucella* species causing brucellosis in pinnipeds (such as seals, sea lions, and walruses). *Brucella* strains have also been isolated from several marine mammals, including common seals, porpoises, common dolphins, bottlenose dolphins, white sided dolphins, striped dolphins, minke whales, Pacific harbour seals, ringed seals, harp seals, and European otters (Forester et al. 2007). Symptoms vary from asymptomatic to observed cases of orchitis, abortion, and meningeoenephalitis (MacDonald et al. 2006).

Human infection has also been reported in individuals working closely with marine mammals (Brew et al. 1999). It is reported that brucellosis in humans from marine mammals is similar to the disease contracted from terrestrial animals with symptoms including headaches, malaise, severe sinusitis, seizures and spinal osteomyelitis (MacDonald et al. 2006). As these species are a new area of research, more studies must be performed to determine their ultimate threat to public health.

Human Brucellosis

Brucellosis in humans is a disease that has the potential to affect several systems with symptoms ranging from mild to severe. Transmission is normally from infected animals. Incubation periods may vary from weeks to several months before symptoms fully develop (Franco et al. 2007). Infected individuals may experience undulating fever, fatigue, and headaches, as well as joint and back pain. These symptoms may have long-term or chronic effects in some patients. More serious symptoms are observed in cases where the bacterium has migrated to the central nervous system or endocardium, in which case meningitis, endocarditis and psychoneurosis can occur (Franco et al. 2007, Alapin 1976, Harris et al. 1954). Although brucellosis is not typically considered a fatal disease,

human cases left untreated can result in mortality (Park et al. 2007, Franco et al. 2007). It is believed that many cases of human brucellosis remain undiagnosed and thus unreported. This may be due to the similarity of initial symptoms to those of influenza (Chain et al. 2005).

Human brucellosis is diagnosed using serological testing and by culturing bacteria from blood, lymph, or cerebrospinal fluid (Seleem 2009). Disadvantages of this method include the slow growth of *Brucella* in culture and a potentially low number of colony forming units (CFU) present in clinical samples due to the stage of infection or to the use of antibiotics prior to sample collection (Seleem 2009, Franco et al. 2007). The Rose Bengal test is also useful for human diagnosis, as well as specially designed ELISAs (Acha et al. 2003, Orduna et al. 2000). The tube agglutination test was the first test used for diagnosis of brucellosis in humans and was later adapted for use in animals. In this test, sera is diluted and added to a tube containing a standard quantity of killed *Brucella* cells. The occurrence of clearing and agglutination following incubation is considered a positive result (Beran 1994). Wright's serum agglutination test, which measures the titer of anti-brucella antibodies (Mert et al. 2003), and Huddleson's slide agglutination test, in which serum agglutination can be rapidly detected (Spink 1956), are successful diagnostic tools for brucellosis. Studies surrounding the use of molecular-based diagnosis using the Polymerase Chain Reaction (PCR) have also been explored with varied results (Kattar et al. 2007). It is also crucial to obtain a detailed case history of any travels to endemic countries or ingestion of any untreated animal products imported from endemic countries.

Treatment for human brucellosis is most commonly through combined antibiotic regimens, as treatments using single antibiotics have shown higher relapse rates (Pappas et al. 2005 & 2006, Solera et al. 1997). The recommended combination is doxycycline coupled with rifampin, but doxycycline with streptomycin or gentamicin have also been effective treatments (Seleem 2009, Solera et al. 1997). Prior to 1986, the World Health Organization (WHO) reported that a doxycycline-streptomycin regimen was the preferred method of treatment for brucellosis in human adults. Currently, however, the WHO Committee on Brucellosis has updated their recommendation to a combined oral treatment of 600-900 mg of rifampicin daily coupled with 200mg/day of doxycycline for a period of 6 weeks for the treatment of acute brucellosis in human adults (WHO 2004). Regardless of therapeutic regimen used, relapse rates of approximately 5-10% have been observed in both adults and children treated for brucellosis (Hall 1990).

Caprine Model for Brucellosis

The goat serves as the primary host for *B. melitensis*, which is the most pathogenic species in humans, and as a secondary host for *B. abortus*, which produces clinical symptoms similar to those observed in cattle. Because both species pose a threat to public health and agriculture, the development of new and more efficient vaccines are an important area of research. There is no single vaccine effective against both species that is safe to use at any stage of gestation (Elzer et al. 2002). Some commonly used vaccines, such as S19 and Rev.1, conflict with serological diagnosis, becoming an obstacle for efforts to eradicate the disease. The development of a caprine model for the study of improved vaccine candidates and other genetic mutants was proposed by Elzer et al.. in 2002.

The use of the caprine model was a logical choice for the study of modified *Brucella* strains. As compared to the commonly used murine model, the goat is a natural ruminant host (Elzer 2002). The goat model is also advantageous over the bovine model due to its lower cost and decreased gestation periods. Furthermore, these attributes allow larger cohort sizes for increased statistical significance (Elzer et al. 2002). The model focuses first on bacterial colonization, monitoring of both pregnant and non-pregnant female animals, as well as the fetus and kid, following conjuctival administration. The colonization assay is performed using various tissue samples obtained at necropsy for bacteriological and serological analysis (Elzer et al. 2002). In this way, the propagation of the pathogen throughout the animal can be monitored. A pathogenicity assay was developed to study the colonization capability of experimental mutants on the dam and subsequently the fetus (Elzer et al. 2002). To test vaccine efficacy, a third assay was developed using non-pregnant females given an experimental vaccine followed by impregnation and a challenge infection at approximately 110 days gestation with virulent *B. melitensis* 16M and/or *B. abortus* 2308 (Elzer et al. 2002).

Brucella melitensis in the Caprine Host

B. melitensis infects the goat as its primary host and can be quickly transmitted from infected dams via vaginal discharges, fetal fluids, placenta, and the fetus itself. Goats are capable of shedding the bacterium for 2-3 months post-parturition in vaginal discharges. There is also long-term shedding observed in milk (Iowa State University, 2009). The usual route of infection is through contact of any of these infectious tissues with mucous membranes. The outcome of exposure varies dependant on the age and health condition of the animal as well as the number of invading bacteria (Alton 1990).

Once infected, animals may experience abortions in pregnant females and impaired fertility in males. Non-pregnant animals are often asymptomatic (Iowa 2009). It has been observed that abortions occur in 70-100% of infected pregnant females; and of those, 90-100% of the dam/kid pairs are found to be culture positive (Elzer et al. 2002). Kids that are not aborted are normally weak at birth and found to be colonized with *B. melitensis*. Elimination of the infection is most easily accomplished by slaughter of the herd (Merck 2006, Alton 1990).

Brucella abortus in the Caprine Host

The primary hosts for *B. abortus* are cattle, but an infection in goats (a secondary host) resembles bovine brucellosis in clinical symptoms and provides a model for the study of *B. abortus* in cattle (Anderson et al. 1986, Meador et al. 1986 & 1989, Elzer 2002). Abortion and colonization are not as prevalent in caprine *B. abortus* infections with an approximately 30-50% abortion rate in infected females and a 50-70% colonization rate in dam/kid pairs (Elzer et al. 2002). It has been observed that both nursing and non-nursing females shed the bacterium, but non-nursing females tend to have higher concentrations of *B. abortus* in their milk (Meador et al. 1986). The colostrum of infected animals tested positive for anti-brucella antibodies, which have been shown to pass to previously antibody-negative neonatal kids following colostral intake (Meador et al. 1986 & 1989).

The serological and clinical pattern observed in the caprine host due to *B. abortus* infection is sufficient for the study of *B. abortus*-derived vaccines in the goat model. Serologically, *B. abortus* RB51 does not revert from a rough to smooth phenotype in the goat, does not promote the formation of anti-OPS antibodies that may interfere with

diagnostic tests, and is capable of inducing the production of anti-*Brucella* antibodies to rough antigens (Shurig et al. 1991, Roop et al. 1991). Clinically, RB51 does not induce abortions in the caprine host when fetuses are injected during the final 50 days of gestation (Roop et al. 1991). RB51 is cleared rapidly from the caprine and ovine host, showing a marked reduction in virulence and survivability when compared to Rev.1 in the these animals. Thus, commercial RB51 has been shown to provide inadequate protection in the caprine and ovine hosts against *B. melitensis* challenge (Adone et al. 2005, Neilson et al. 2004).

Brucella Vaccines

Vaccination against bovine brucellosis, a disease caused by *B. abortus*, is a powerful tool in the effort to eradicate the disease in the United States. The first brucellosis vaccine approved for field use by the US government, smooth *B. abortus* Strain 19 (S19), was approved and implemented in 1941 (Neilson et al. 1990). Conflicts with S19 arose with diagnostic testing and a new rough vaccine, *B. abortus* RB51 (RB51), was approved by the USDA to replace S19 as the standard brucellosis vaccine in 1996 (USDA 1996). Although RB51 resolved the problems presented by S19 with diagnostic testing, the popular opinion of RB51 is that it may be a less efficacious vaccine than S19. The immunogenic differences between S19 and RB51 are principally a manifestation of differences in structure, function, and survivability in the host.

The responses of the host's immune system to a typical infective strain of *B. abortus* include both cell-mediated and humoral reactions. The humoral reactions are those involving the production and function of antibodies that target the surface components of the cell's outer membrane and complement. Studies on the role of

complement have shown it to be ineffective in combating *B. abortus* directly, but it may

aid in phagocytosis (Timoney et al. 1998)(Barquero-Calvo et al. 1997). The primary

immunogenic component of the smooth outer membrane is the O-polysaccharide (OPS)

component of the lipopolysaccharide (LPS). The antibody response to the OPS mediated

by the host is ineffective in providing protective immunity against a challenge infection

as shown through effectiveness of the rough RB51 vaccine strain. In fact, the presence of

IgG1 antibodies predominantly produced in response to *B. abortus* does not correlate to

elimination of the pathogen (Bellaire et al. 2005). However, opsonic antibodies can

promote intracellular killing and slow the reproduction rate of intracellular *B. abortus,*

but antibodies alone are not effective in clearing the pathogen from the host (Timoney et

al. 1998, Arenas et al. 2000, Bellaire et al. 2005). In terms of vaccination, the cell-

mediated immune response is primarily responsible for protective immunity.

The cell-mediated immune response to *B. abortus* is primarily mediated by

activation of phagocytes and T-cells of the adaptive immune response. The role of cell-

mediated immunity is to identify and destroy host cells housing harmful intracellular

pathogens, such as viruses and facultative or obligate intracellular bacteria (Abbas 2005).

For *B. abortus*, the macrophage is primarily responsible for ingestion and clearance of the

pathogen from the extracellular environment. Macrophages that are not activated prior to

ingestion of *B. abortus* differ in their interaction with the pathogen than do previously

activated macrophages, which are considerably more brucellacidal (Barquero-Calvo et al.

2007, Elzer 2007). Antigen presentation to both CD4 and CD8 T-cells is a key

occurrence in the host's development of protective immunity. *B. abortus* ingested by

inactivated macrophages can inhibit development of the phagolysosome or neutralize the

acidic environment therein, avoid killing, and eventually replicate within the cell

relatively undetected (Barquero-Calvo et al. 2007). In these inactivated phagocytes, the

pathogen causes the macrophages to become immunosuppressive against a strong CD4+

Th1 response (Forestier et al. 2000). However, previously activated, or immune,

macrophages degrade *B. abortus* upon ingestion and phagolysosomal fusion, where it can

be recognized and presented to CD4+ Th1 cells by the MHCII receptor. An effector

CD4+ Th1 cell further activates macrophages by secreting IFNγ, which triggers an

increase in antimicrobial activities against the pathogen. Likewise, *B.abortus* elicits a

strong CD8+ T cell response, which mediates cytotoxic activity and functions to lyse

infected cells. If a macrophage that houses replicating *B. abortus* cells is lysed, the

pathogen is released into the extracellular environment, where it can either infect other

cells or become ingested and destroyed by activated macrophages (Elzer 2007, Baldwin

and Winter 1994, Jones 1992, Araya et al. 1989).

Brucella abortus S19

The S19 vaccine strain was developed in 1923 by Dr. John Buck and was shown

to produce adequate immunogenicity in guinea pigs without causing detrimental

pathology. Dr. Buck found that S19 was similarly effective in cows against challenge

infections when given in the correct dose. Statistically, S19 provides 70-90% protection

in pregnant heifers in preventing abortion and infection (Nicoletti 1990). This attenuated

strain was created by isolating a virulent *B. abortus* strain from an infected animal which

was then serially-passaged and allowed to incubate at room temperature for several

months. When examined, Dr. Buck found the isolate to be attenuated (Graves 1943).

However, it has also been observed that S19 may cause abortions in pregnant cows,

orchitis in bulls, pyrexia, and other infections in some cases (Nicoletti 1990). Human

infections have also been reported through accidental vaccination or exposure (Wallach

et al. 2008). Antibodies to S19 can be passed from cow to calf through the colostrum

following birth. If calves are vaccinated during this period, these maternal antibodies are

capable of neutralizing or killing S19. Vaccination of cattle herds is typically performed

in 3 to 6 month old heifers at a standard dose of 5×10^{10} to 8×10^{10} CFU (OIE 2009,

Poester et al. 2006).

The immunologic response to S19 is similar to that of an infectious strain of *B.*

abortus, but does not result in a full infection (Nielson et al. 1990). S19 is a smooth

strain of *B. abortus* and expresses a fully intact and functional LPS with the OPS on the

surface of the outer membrane. Because the OPS is a primary immunoantigen, S19 is

capable of inducing the production of OPS-specific antibodies in the host, stimulating

both cell-mediated and humoral immunity. However, this feature of S19 is problematic

since standard diagnostic tests detect the presence of anti-OPS immunoglobins as a false-

positive reaction. Typically, S19 will provide protection against virulent *B. abortus* for

several years while the presence of anti-OPS antibodies will decrease within months of

vaccination. However, it has been observed that some animals become infected

permanently with S19 following vaccination, continuing to produce anti-OPS antibodies

for years following vaccination (Jacob et al. 2005). For these chronic shedders, serologic

tests will constantly provide positive results for vaccinated animals that are uninfected.

Because of this issue, a new vaccine, named RB51, was licensed in 1996 that was

a rough strain of *B. abortus* 2308 (USDA 1996). Because RB51 lacks the OPS found on

virulent strains of *B. abortus* as well as on the S19 vaccine strain, the problem with

diagnostic tests was solved. RB51 does not promote the production of anti-OPS antibodies in a strong humoral response, yet is effective in providing adequate protection against challenge infections (Poester et al. 2006).

B. abortus RB51

The current U.S. vaccine used to protect cattle against bovine brucellosis is the *B. abortus* RB51 vaccine strain. RB51 was developed by making serial-passages of *B. abortus* 2308 on rifampicin and penicillin supplemented Tryptic soy agar (TSA) plates (Schurig et al. 1991). The resultant RB51 strain was rifampicin-resistant and lacked the OPS of the original smooth 2308 strain. Genetically, this is due to a defect in the LPS biosynthesis loci (Vemulapalli et al. 2004). Although there was concern surrounding the release of an antibiotic-resistant strain of *B. abortus*, in February of 1996 the United States designated RB51 as the premier vaccine, replacing the Strain 19 vaccine used prior to this switch (Poester et al. 2006).

RB51, which lacks the O-polysaccharide chain normally found on strain 19 and wild-type *Brucella abortus,* does not initiate the production of smooth OPS antibodies in the vaccinated animal. Thus, RB51, unlike strain 19, does not produce a positive test result for an animal that is negative for brucellosis but has been exposed to *B. abortus* through vaccination. It has also been shown that RB51 does not revert to a smooth phenotype when administered. RB51 has also been shown to be equally efficacious as strain 19, but is less abortigenic and does not produce any symptoms of disease after vaccination (Poester et al. 2006) (Perry 2005). RB51 does not produce a very strong antibody reaction in the vaccinated animal but it does cause a cell-mediated response that is the primary factor in protecting the animal from a full infection (Poester et al. 2006).

Studies have shown that RB51 is 70-90% effective in preventing abortions and disease in cattle tested (Stevens et al. 1995) (USDA 1996). Currently, RB51 remains the principal vaccine for bovine brucellosis at this time and an important component of the State-Federal Brucellosis Eradication Program. Domestic calves are vaccinated with RB51 at 4 to 12 months of age at a dosage of $1.0\text{-}3.4 \times 10^{10}$ CFU and tagged appropriately (USDA 1996, Poester et al. 2006). Following vaccination, RB51 is quickly cleared from the draining lymph nodes (Chevelle et al. 1992 & 1993). Over the course of a typical vaccination with live attenuated RB51, the organism is typically cleared from cattle in 6 to 8 weeks. RB51 is usually undetectable in the blood stream within a period of 3 days and are not present in mucosal secretions (USDA 1996). Both subcutaneous and conjunctival administrations of RB51 have been proven effective in conferring protection against challenge infections of *B. abortus* (Jiménez de Bagüés et al. 1994, Stevens et al. 1996), and experiments focused on oral vaccination have yielded promising results (Elzer et al. 1998).

Brucella melitensis Rev.1

The Rev.1 vaccine was developed in 1957 by Elberg and Herzberg for the protection of goats and sheep against *B. melitensis* and is a streptomycin-non-dependent reverse mutant of the streptomycin-dependent *B. melitensis* 5056, a virulent strain (Sanmartino 2005). Low virulence has been observed in small ruminants when the vaccine is administered under standard conditions. Furthermore, upon continued passage, Rev. 1 does not revert to a pathogenic form (Diaz-Aparicio 2004). Similarly to S19, Rev-1 is a smooth strain with an intact OPS. Thus, Rev.1 vaccinated animals produce antibodies identical to animals naturally infected with wild-type *Brucella*, which

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may produce false positive results using standard serological tests.

When administered to pregnant animals, Rev.1 has been shown to be abortigenic at standard vaccination doses. It is also found that a reduced dosage of Rev.1 does not fully prevent the induction of abortion and does not confer full protection against *B. melitensis* infections in both goats and sheep (Gonzalez et al. 2008, Blasco 1997). Because of the pathogenicity issues when administered to pregnant animals, there is no completely safe strategy for vaccination using Rev.1. Conjunctival vaccination of sheep during lambing season or late lactation has been suggested as an optimal vaccination strategy for adequate protection with a reduced frequency of abortion (Blasco 1997).

The recommended vaccination method for Rev.1 is to use a standard dose (1×10^9 to 2×10^9 CFU) subcutaneously injected for female goats and sheep at the age of 4 to 6 months old. Following vaccination, Rev.1 causes infection in the animal for a period of 3 months, with colonization generally restricted to the lymph nodes and spleen. The organism has also been located intracellularly in the dendritic follicular cells and macrophages of the draining lymph nodes following vaccination (Munoz 2008). Strain persistence has also been reported due to horizontal spread among vaccinated herds (Banai 2002, Bardenstein et al. 2002). Rev.1 induces a potent antibody response, which poses an obstacle to diagnostic serological tests (Blasco 2006). Reduced serological responses have been observed in animals administered Rev.1 through conjunctival vaccination, as opposed to subcutaneous injection.

Other Vaccine Research

In attempts to increase the safety of vaccination, studies to develop a vaccine that is protective and non-pathogenic in animals, as well as non-infectious to humans were

conducted.   Vaccines produced from killed field isolates and laboratory strains have met

with mixed reviews. Although some vaccine candidates decreased pathogenicity,

protection provided was inadequate (Schurig 2002). Furthermore, in killed vaccines

using smooth strains of *Brucella*, positive serology poses a problem to detection

capabilities using standard diagnostic tests. Vaccines developed using antigenic fractions

of *Brucella* cells, including portions of the *Brucella* cellular envelope, outer membrane

proteins, and LPS fractions, among other proteins, have been tested. Some vaccines

utilizing antigenic fractions, when administered using an immunogenic adjuvant, have

provided immunity in laboratory tests (Shurig 2002). DNA vaccine research for

brucellosis is limited to small laboratory animals and has not been examined for use in

natural hosts. In these studies, the level of protection provided by DNA vaccines is

inferior when compared to the live-attenuated vaccines currently in use (Schurig 2002).

Nevertheless, there is no killed or sub-unit *Brucella* vaccine approved for commercial

use.

      Human vaccine trials

      There is no vaccine against any species of *Brucella* that is safe for human use.

The vaccines previously described for use in animals are infectious to humans, and some

carry antibiotic resistance genes that are a cause of concern when considering treatment

methods. In developing countries in which brucellosis is rampant, human vaccine trials

have been performed with little success. Attenuated live vaccine strains administered to

humans show varied protective capabilities and adverse side-effects. Human vaccine

trials using sub-unit brucellosis vaccines also provide varied results (Schurig 2002). The

development of a vaccine for human use in countries where the disease has been eradicated is also of interest considering the status of brucellosis as a bioterrorist threat. It is generally accepted, however, that the best prevention technique for brucellosis in humans is through eradication of the disease in animals and proper treatment of food products intended for human consumption.

Brucella Genomic Studies

There are currently ten fully sequenced *Brucella* strain genomes available from five species of Brucellae (*B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, and *B. canis*). Twenty five additional Brucellae genomes are also in the progress of being sequenced (Seleem et al. 2009). Complete genomic sequences allow researchers the opportunity to study and manipulate genes of *Brucella* spp. and their protein products in order to learn more about the functional aspects of virulence, replication, and survival of this organism, both in and out of the host. Not only does this provide insight into the genetic coding sequences unique to brucellae, but it also facilitates the studies of conserved coding sequences and their comparative functions across several species of bacterial pathogens. Genomic studies have also facilitated proteomic studies to identify proteins involved in virulence, pathogenicity, and host specificity. Expanding genomic data has provided new perspectives into the examination of the *Brucella* genus as a whole, as well as comparative studies between *Brucella* species. Manipulation of the *Brucella* genome has permitted the determination of the function of several genes. Genes involved in host specificity, survival, and virulence are frequently targeted in efforts to improve vaccination. Techniques such as genetic transformation or gene disruption in *Brucella*

spp. are widely used to study specific genes with successful results (Zygmunt et al. 2006).

Molecular transformation is a method in which the natural characteristics of an organism can be manipulated by altering its genetic repertoire. This technique has been used frequently in the study of genetic complementation in brucellae. Methodology includes the construction of a plasmid vector containing a selective marker and the gene region of interest, transformation of the vector into the cell, and selection of genetically modified cells. The pBBR1MCS plasmid is a broad-host-range vector that is stably maintained in *Brucella* species, under both *in vivo* and *in vitro* conditions (Elzer et al. 1995). The vector is retained at a low copy number within the cell, but is not integrated into the genome (Kovach et al. 1995, Elzer et al. 1995). Variations of the pBBR1MCS plasmid contain different selectable markers used for the identification of effectively modified organisms.

Other methods frequently used in genetic manipulation include Gateway-based destination vectors and gene disruption or deletion. The Gateway recombination cloning system is used to move genes into a multiple vector system for functional analysis and protein expression without the use of the restriction endonucleases and ligases used in traditional cloning methods (Invitrogen 2003). This system has been used to identify and study open reading frames of *B. melitensis* and *B. abortus* at a proteomic level to determine biological function (Dricot et al. 2004, Hallez et al. 2007).

Suicide vectors, also known as gene-replacement plasmids, are utilized for site-directed chromosomal insertion by homologous recombination between homologous DNA sequences. The vector itself does not replicate and is not maintained in the cell

(Elzer et al. 1995). Gene-replacement plasmids are introduced via electroporation or chemical treatment into the cell, similar to the methodology described previously. The goal is to replace a target gene of interest within the genome with a gene from the vector, most often carrying a selectable marker for the detection of modified cells (Sherratt 1995). The marker present on the vector is flanked with sequences homologous to those flanking the wildtype gene of interest within the genome. After introduction into the cell, the wildtype gene of interest is disrupted, following a double-crossover homologous recombination event which incorporates the selectable marker into the genome (Sherratt 1995). This method is a form of site-directed mutagenesis.

Another method of gene disruption is through transposon mutagenesis. This technique uses random gene inactivation to identify cellular functions associated with virulence and survival (Wu et al. 2006). This form of mutagenesis allows the random transference of genes, most often selectable markers, into the chromosome of a host organism using transposable elements, potentially disrupting the wildtype gene located at the insertion site. A pool is created from those mutants selected to have taken up the selective gene carried by the transposon. This mutant pool is then introduced into a host animal and analyzed for reductions in virulence as compared to the wildtype strain. This method of mutagenesis is randomized and not directed to specific genes.

Gene disruption or deletion mutants obtained using the methods above are analyzed *in vivo* with regard to reduced survival and intracellular replication to determine the function of the particular gene in question. Studies in *Brucella* species using gene disruption or deletion mutants have proven successful in examining specific genes of

interest (Halling et al. 1991, Tatum et al. 1993, Drazek et al. 1995, Edmonds et al. 2001 & 2002, Wu et al. 2006).

Hemagglutinins

Blood agglutinins are produced by many pathogens, including bacteria, viruses, parasites, and plants (Nelson et al. 2006). Much of the current research performed on pathologic hemagglutination is focused on viral hemagglutinins, namely the influenza virus. There are two primary functions of viral hemagglutinins in infection: to recognize the host cell via sialic acid-associated receptors and to facilitate entry of the viral genome into the target host cell (Gambrian et al. 2006, Suzuki 2005, White et al. 1997).

In historical studies conducted under *in vitro* conditions, it was discovered that some bacteria produce proteins that promote the agglutination of red blood cells (Netter et al. 1954). The bacterial hemagglutinin has been proposed to function in adherence of the organism to the surface of host cells (Alam et al. 1997). Direct bacterial hemagglutination occurs when the protein hemagglutinin itself causes the agglutination of erythrocytes (Neter et al. 1954). This was first described by Kraus and Ludwig in 1902 (Koransky et al. 1975). Indirect hemagglutination occurs when the bacteria increase the liability of the RBC to agglutination by antibodies by bringing about a physiological change in the surface properties of the erythrocyte (Neter et al. 1965).

Although many proteins have been studied as hemagglutinins, the lipopolysaccharide (LPS) has been the focus of many bacterial hemagglutination experiments (Alam et al. 1997, Watkins et al. 1987). The LPS of *Brucella* species serves several functions in virulence and survival of the pathogen, yet rough strains are also capable of virulence and survival *in vivo*. Furthermore, there is no data that shows the

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Brucella LPS as being involved in invasion of the host cell (Rocha et al. 1999, Aragon et al. 1996). However, there is data that shows an increased rate of adherence and uptake into host cells for rough strains of *Brucella* as compared to smooth strains, yet rough strains were not as efficient to replicate within the host cells (Ferrero et al. 2009). This suggests an adherence mechanism separate from the LPS. The mechanisms concerning the association of *Brucella* to host epithelial cells, RBCs or the effector molecules involved in cellular uptake are not clearly understood. Since cellular adherence is necessary for uptake of facultative intracellular pathogens, it is important to understand the mechanisms and molecules involved in this process. Many studies have been performed to identify and characterize virulence factors produced by *Brucella* species that are associated with the attachment and invasion of host cells, yet exact mechanisms remain unclear (Rosetti et al. 2009, Ferrero et al. 2009, Castaneda-Roldan et al. 2006 & 2004, Rocha et al. 2002 & 1999, Guzman-Verri et al. 2001, Zaitseva et al. 1996, Eskra et al. 1991, Hoffman et al. 1990, Vendrell et al. 1990).

An increase in the identification and study of virulence genes for *Brucella* species has occurred since the mapping of several *Brucella* genomes. In 2002, DelVecchio et al. described a gene coding for a putative hemagglutinin (GI:17989062) found in the genomic sequences of *B. melitensis* and *B. ovis* (DelVecchio et al. 2002a &2002b). A highly homologous but not identical gene sequence is also found in *B. suis* and *B. canis*. Other matches with some homology to the *B. melitensis* putative hemagglutinin gene are as follows: a *B. ovis* hemagglutinin (GI:148558421); a *B. melitensis* glycoprotein X precursor (GI:225686341); a cell wall protein AWA1 precursor in *B. suis* (GI:254702929), *B. canis* (GI:161620616), *B. ceti* (GI:254715437), and *B. pinnipedialis*

(GI:254708522); and a putative cell wall surface protein of *B. ovis* (GI:148558357) and *B. suis* (GI:23500299). The gene is not naturally found in any strain or subspecies of *B. abortus*.

Previous studies were performed on suspected *Brucella* hemagglutinins and their agglutinative properties on human and animal erythrocytes. It was shown that *B. abortus* 2308 has a significantly lower titer using the hemagglutination test, as described by Evans et al. (1980), than *B. melitensis* 16M. Furthermore, the previously described *B. melitensis* hemagglutinin (GI:17989062) was investigated in the caprine model. The gene was placed into *B. abortus* 2308 via the pBBR1MCS-4 plasmid (tagged 2308-QAE) and a gene deletion *B. melitensis* 16M mutant was also created. The *B. abortus* 2308-QAE strain showed increased virulence (as compared to *B. abortus* wild-type) in colonization and pathogenicity studies, similar to the results in the *B. melitensis* 16M challenge animals. The 16M deletion mutant showed no signs of attenuation in colonization of a caprine host, but the pregnant animals that received the deletion mutant showed a 30% reduction in abortions when compared to the parent *B. melitensis* strain. It was concluded that the specific QAE gene was a putative hemagglutinin and virulence factor of *B. melitensis,* as well as a possible host specificity factor for the caprine host (Perry 2007).

Protein Characterization

Identification of protein characteristics is an integral part of learning the functional aspects of a protein as well as the role it plays in cellular activity and survival as a whole. Protein expression and function may vary depending on *in vivo* or *in vitro*

conditions, making it necessary to examine both avenues in characterization of the same protein.

For assessment of protein expression *in vivo*, cellular activity inside of a living animal host is examined. The identification of protein-specific antibodies using western immunoblot techniques and absorption assays are useful in determining the relative size and antigenicity of a specific protein. Antibody absorption assays examine immune sera by combinination with lysates of the *in vitro*-grown pathogen to identify antibodies specific for proteins exclusive to expression *in vivo* by removal of cross-reacting antibodies (Handfield et al. 2000). Specifically, the serum of an animal that has been exposed to a protein of interest via infection or vaccination and, in theory, contains *in vivo*-produced, protein-specific antibodies is mixed with lysates from cells lacking the gene for the protein of interest, but otherwise identical to the *in vivo* strain. This step serves to remove any cross-reacting antibodies in the serum (Handfield et al. 2000, Rollins et al. 2005). Next, the absorbed serum can be subjected to analysis by western immunoblot containing cellular lysates of both the *in vivo* strain and the strain used for absorption. Following identification by secondary antibody conjugation and blot development, coupling of *in vivo*- produced antibodies to the protein of interest can be analyzed to gain further information on the specific protein. This type of antibody absorption assay is useful if the *in vivo* expressed protein of interest is sufficiently antigenic to induce the production of antibodies adequate for detection using western immunoblot (Handfield et al. 2000, Rollins et al. 2005). Specifically, this assay is most useful in the examination of externally exposed or secreted proteins.

For examination of proteins *in vitro*, cells are grown outside of an animal host in or on laboratory-made media. There are numerous tests available to examine proteins produced under *in vitro* conditions.   Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 2D gel electrophoresis followed by mass spectrometry analysis are commonly used for the characterization of such proteins.

SDS-PAGE is a commonly used biochemical technique used to separate proteins based on molecular weight. Proteins are separated on a polyacrylamide gel via electrophoresis, during which an electric current is applied to the gel, causing negatively charged proteins to migrate at different rates depending on the size of each protein (Shapiro et al. 1967). Following protein separation via electrophoresis, the gel is stained for visualization of the separated proteins. A common stain, Coomassie brilliant blue, is commonly used due to its exceptional sensitivity to protein binding (Merril 1990). Once stained, the separated proteins can then be examined based on molecular weight.

Analysis using 2D gel electrophoresis is a technique that separates proteins on two dimensions: molecular weight and isoelectric point. This technique provides a greater degree of protein separation than SDS-PAGE. 2D gel electrophoresis is also considered to be the most sensitive procedure for the analysis of low abundance proteins (Berth et al. 2007). This technique is commonly used to identify unique proteins between two strains of highly homologous organisms. To achieve this, lysates from each strain are subjected to 2D gel electrophoresis and examined, following staining, to locate any unique proteins not shared by both strains. Further characterization of these unique proteins can be assessed by mass spectrometry analysis, during which proteins are ionized and introduced to a mass analyzer (Hernandez et al. 2006, Zhang et al. 1994).

Proteins can be identified based on amino acid sequence and protein quantity can be assessed.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a method used to amplify specific regions of DNA from a large amount of diverse sequence for the purpose of visualization and identification. PCR is widely applied in many different fields of science including biochemistry, molecular biology, genetics, medicine and forensics. Because of the sensitive nature of PCR, the workspace and technique must be both sterile and precise for the prevention of contamination. However, there are several inhibiting factors for PCR in even the most sterile conditions. PCR is currently being studied as a possible detection method for brucellae in bodily fluids such as blood and urine (Navarro et al. 2006, Queipo-Ortuno et al. 2006, Zerva et al. 2001, Morata et al. 1999, Yagupsky 1999). Currently, there is no accepted standard for detection of brucellae using PCR and detection is most often accomplished using tissue and blood cultures.

The current method of diagnosing brucellosis involves culturing the organism from the blood, bone marrow or tissues or testing the animal for the presence of anti-OPS antibodies (Yagupsky 1999). A standing problem with culture, most often from the blood, is that *Brucella* are very slow growing organisms and several days are needed for a positive confirmation, which can delay treatment. Standard diagnostic testing using antibody recognition is also problematic due to false positives caused by *Y. enterocolitica* 0:9 infections and smooth vaccine strains. In endemic countries where brucellosis is a serious threat to public health, the development of a more rapid yet still reliable method for diagnosis would greatly detection and treatment methods. A PCR technique for the

detection of *Brucella* species has many advantages over current methods. The PCR

technique itself is fairly easy to perform, affordable, and data can be acquired in less than

one day. Other advantages include the targeting of brucella-specific DNA and the

independence of antibody-based testing, which largely reduce the potential for cross-

reaction-based false positive results.

Currently, there is no standardized method for diagnosis of brucellosis using PCR

and detection capabilities have varied between labs (Elfaki et al. 2005, Navarro et al.

2004, Bricker 2002). Some studies have shown that diagnosis using PCR techniques

have had equal or better detection capabilities as blood culture, yet provided results in

only hours, as compared to days for culture (Navarro et al. 2006, Queipo-Ortuno et al.

2006, 2005 & 1997, Zerva et al. 2001, Morata et al. 1999). Conversely, there have also

been studies in which PCR techniques have provided inferior detection capabilities in

comparison with culture techniques (Maas et al. 2007, Kattar et al. 2007, Navarro et al.

2004 & 1999). Reasons for inconsistent results may lie in inconsistencies involving

techniques used among labs and in the variability of fluids tested. Many labs perform

PCR tests using whole blood cultures, however the heme-components found in whole

blood can inhibit PCR amplification. To circumvent this issue studies using serum were

evaluated, but were ineffectual in some cases if the patient tested was not bacteremic at

the time the sample was taken. Urine has also been tested, yet only two *Brucella* species

are shed in urine and neither is a threat to human health. Other inconsistencies were

found in the extraction of DNA using either manual DNA preparation versus the use of

different commercially available kits, periodic testing versus one-shot testing, and

differences in primers used. The most commonly used primer in PCR detection studies

was developed by Baily et al. in 1992 for the detection of *B. melitensis* and *B. abortus* (Baily et al. 1992). While successful trials have been reported in the use of the Baily primers, other primers are also of interest in PCR techniques for *Brucella* detection. Of note are the AMOS primers developed by Bricker et al., which are capable of differentiating strains of *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis* by conventional PCR and gel electrophoresis (Bricker et al. 1994). Studies using the AMOS primers have provided promising results (Bricker et al. 2003, 1995). In some cases, the inability to reproduce previously successful PCR techniques by other labs has been noted (Navarro et al. 1999).

HYPOTHESIS

The hypothesis for this study is that region E putative protein is a cell-surface hemagglutinin and host specificity factor that, when expressed *in trans* through *B. abortus* RB51 strain pQAE (RB51-QAE), will promote transient colonization of the host and elicit an immune response greater than the parental RB51 without producing any signs of adverse pathology of in the goat model. The modified strain of *B. abortus* RB51-QAE was analyzed using an *in vivo* colonization study in the goat model and *in vitro* experiments focused on hemagglutination, immunogenicity, and protein characterization.

A second hypothesis for this study proposes that conventional PCR methods are sufficient for the detection of both *B. melitensis* and *B. abortus* colony forming units in whole blood, plasma, serum, and urine. This hypothesis was tested using uninfected whole blood, plasma, serum, and urine to create and test serial dilutions of *B. melitensis* 16M and *B. abortus* 2308 using the Omp25 and Baily primer sets, designed for the detection of all *Brucella* spp.

CHAPTER ONE

COMPARISON OF *BRUCELLA ABORTUS* RB51 AND RB51 CONTAINING PQAE IN THE CAPRINE MODEL

Introduction

Vaccination against brucellosis is an integral tool in efforts to control the disease worldwide. The current vaccine used to protect cattle against bovine brucellosis is *B. abortus* RB51, which lacks the OPS and does not interfere with serological testing (USDA 1996). As a rough vaccine, RB51 does not produce a very strong antibody reaction in the vaccinated animal; but it does induce a cell-mediated response for protective immunity. RB51 provides limited protection in goats and little to no protection in other animals, such as elk and bison, which serve as major U.S. reservoirs for *B. abortus* in the wild (Moriyon et al. 2004). The Rev.1 vaccine has proven more effective in the caprine host, but promotes antibody formation that interferes with diagnostic testing. Development of an improved vaccine that is safe, effective, and does not impede standard diagnostic testing could serve as an important advancement in eradicating the disease globally.

Plasmid QAE (pQAE) was created to study a putative hemagglutinin found in *B. melitensis*. The QAE plasmid consists of the pBBR1MCS-4 plasmid (which contains an ampicillin resistance marker) and a region (designated region E, GI:17989062) from the genome of *B. melitensis* that is hypothesized to contribute to virulence or host specificity. The development of pQAE was accomplished by comparing the genomes of *B. abortus* 2308 and *B. melitensis* and finding gene regions that differed. Once a unique region was found in the genome of *B. melitensis*, this region was investigated, isolated, and cloned

into pBBR1MCS, and then electroporated into *B. abortus* 2308 (Perry 2007). This new

strain of 2308 (2308-QAE) was then used to infect pregnant goats. The results of this

experiment yielded approximately the same results seen for goats infected with *B.

melitensis*, indicating that the region of the *B. melitensis* genome that was carried by

pQAE and transferred to 2308 was effective in increasing host specificity (Perry 2007).

In addition, the effects of pQAE expression by *B. abortus* RB51 in the caprine

host were examined. Animals were conjunctivally inoculated with either RB51 or RB51

expressing pQAE (RB51-QAE) and tissue colonization levels were monitored over a

period of 42 days post-inoculation. It was expected that RB51-QAE would remain

attenuated, but survive longer in the tissues, potentially producing a greater immune

response and thus, a more broadly efficacious vaccine.

Successful trials with RB51-QAE may ultimately lead to the development of a

new, more effective vaccine that will serve as a better tool in the eradication of

brucellosis than the currently used RB51 vaccine. Due to the worldwide problem with

this pathogen and the need for an alternative vaccine, a caprine brucellosis model system

was developed for testing promising vaccine candidates (Elzer 2002). Using this

protocol, a vaccine candidate that incorporates pQAE into RB51 was tested. The goal of

this study was to compare the modified RB51 strain (RB51-QAE) with the parental strain

for colonization levels and pathological effects.

Materials and Methods

Creation of B. abortus RB51-QAE

A 100 µl dose of the *B. abortus* RB51 (Colorado Serum) parental strain was

plated on a brucella selective, blood agar (BS-BA) plate and incubated at 37°C in a 5%

CO_2 atmosphere for approximately 72 hours. All resultant colonies were harvested and used to inoculate 25 ml of sterile Brucella broth. The cells were incubated overnight in a shaking water bath set at 37°C.

The culture was then equally divided by placing 1 ml of the total culture into each of twenty-five 1.5 ml microcentrifuge tubes. These cells were pelleted, the supernatant discarded, and the pellets were combined into a single microcentrifuge tube. The pellet was washed a total of 5 times in sterile dH_2O then re-suspended in 100 μl of cold, sterile dH_2O.

For electroporation, 33 μl of the suspended RB51 cells were combined with 3 μl of cold pQAE plasmid solution in an Eppendorf Electroporation Cuvette. The cuvette was then placed in an Eppendorf 2510 Electroporator set at 2.5kV and was electroporated for 5.6 ms. Immediately after electroporation, the cells were supplemented with 500 μl of cold, sterile SOC-B recovery media. The cells were then left overnight in a shaking water bath set at 37°C.

The next morning, BA plates containing 100 μg/ ml ampicillin were used to spread 100 μl of the electroporated RB51-QAE cells. These plates were incubated for a period of 2 weeks in a 37ºC, 5% CO_2 environment and observed for growth daily after the initial 72 hours.

All of the colonies growing after this 2 week period were suspected to be RB51-QAE colonies and were tested accordingly. All resultant colonies tested positive for oxidase, catalase, and urease tests, which is typical for *B. abortus* colonies. Furthermore, a miniprep plasmid isolation was performed on rando mly selected colonies and the pBBR1MCS-QAE plasmid was identified from these colonies. To detect the presence of

region E in the isolated pQAE DNA, the cells were chloroform killed and a restriction enzyme digestion using *Eco*R V (New England Biolabs) and PCR was performed, both targeting the region E portion of pQAE. Samples of the pure pQAE plasmid, as well as samples obtained through miniprep procedures following tissue culture, were sequenced using BioMMED (Baton Rouge) facilities.

Confirmation of pQAE in B. abortus RB51-QAE

Plasmid isolation was performed using the Qiagen Buffer System kit (Qiagen, Inc.) to verify the presence of pQAE in RB51-QAE cells. A broth culture of RB51-QAE was allowed to incubate at 36°C overnight in a shaking water bath. Two ml of the culture was pelleted by centrifuge at 9000rpm for 5 minutes and the supernatant was separated then discarded. The pellet was then re-suspended in a series of three buffers; 300μl of Bufer P1 (50mM Tris base; 10mM EDTA, pH 8; 100 μg/ ml RNase A), then 300 μl of Buffer P2 (200mMNaOH, 1% SDS), and lastly 300 μl of chilled Buffer P3 (3.0 M CH_3CO_2K, pH 5.5). The tubes were vortexed after the addition of Buffer P1 and mixed by inverting the tubes after the addition of Buffers P2 and P3. The tubes were centrifuged at 14,000 rpm for 10 minutes, after which the supernatant was transferred to a clean 1.5 ml microcentrifuge tube. The volume of the supernatant was measured and an amount of isopropanol 0.7 times that of the total volume was added. The tubes were inverted multiple times to precipitate the DNA and centrifuged for 15 minutes at 14,000 rpm. The supernatant was discarded, the DNA pellet was allowed to air dry, and was then re-suspended in 25 μl of sterile dH_2O.

Following plasmid DNA isolation, a Polymerase Chain Reaction (PCR) amplification targeting the region E gene sequence was performed. The Failsafe™ PCR

System was used for this procedure, specifically with 2X Premix G (Epicentre Biotechnologies). Twelve 2X Premixes (A-L), each containing a specific mix of dNTPs, buffer, and varying amounts of MgCl2 and FailSafe™ PCR Enhancer (with betaine), were subjected to a PCR assay with a control sample of region E DNA.

Reactions of 30µl were prepared for each of the pQAE isolation samples. For each sample, 0.75 units of Failsafe PCR Enzyme Mix, containing a mix of thermostable DNA polymerases, were added with 2X Premix G, 0.2µM of each primer, sterile dH$_2$O, and approximately 300 ng of template DNA. The primers used for this assay, ORF-944F (5'-GAATTGGCGACCTGACTGAGGA- 3') and ORF-944R (5'-CTCACGGCTGTTCTCCTTTAACA- 3'), were designed to target the 1988 bp gene sequence of region E. These primers were designed through The Institute of Molecular Biology and Medicine at the University of Scranton (Scranton, PA) using the *B. melitensis* 16M choromosome II gene sequence (Genbank Accession Number AE008918). The primers were produced by Integrated DNA Technologies, Inc. (Coralville, IA), re-suspended upon arrival using sterile dH$_2$O, and stored at -20°C.

The PCR samples were placed in a MyCycler™ Personal Thermal Cycler (BioRad Laboratories, Inc., Hercules, CA). For the optimal amplification of Region E, an initial denaturation step of 96°C was held for 5 minutes. Each reaction was then subjected to denaturation at 96°C for 1 minute, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes over 30 cycles. Lastly, a prolonged extension phase was programmed for 5 minutes at 72°C. The amplified samples were stored at 4°C until visualization with agarose gel electrophoresis using ethidium bromide staining.

A restriction enzyme digestion was performed using EcoRV (New England Biolabs) on the pQAE miniprep isolations purified from RB51-QAE. The purpose of this procedure was to excise the region E gene sequence (approximately 2kb) from the pQAE plasmid (approximately 4kb) for visual confirmation using agarose gel electrophoresis. Twenty units of EcoRV were used with 2.5μl 100μg/ ml bovine serum albumin (BSA), 2.5μl of 1X NEBuffer3, and a volume of dH$_2$O to bring the total volume to 25μl. The reactions were incubated for 1 hour at 37°C in a shaking water bath under slight agitation. To deactivate the restriction enzyme activity, the samples were placed in a 85°C heating block for 20 minutes.

Agarose gel electrophoresis was used to visualize the DNA products of PCR, restriction enzyme digestion, and plasmid and genomic DNA isolation. A 5μl sample of each DNA preparation was mixed with 5μl of 6X loading buffer (12% Ficoll 4000, 1.0 M Na$_2$EDTA, pH 8, 0.6% SDS. 0.15% bromphenol blue) and loaded onto a 1% agarose gel. Molecular weight standards of either 100bp, 1 kb, or a supercoiled molecular weight ladder (New England Biolabs) were run on each gel to determine the approximate size and concentration of each DNA sample. The gel was made using UltraPure™ Agarose (Invitrogen Corporation) in TAE buffer (Tris-base, Na$_2$EDTA, glacial acetic acid). Each gel was run in a BioRad DNA Sub Cell (BioRad Laboratories, Inc.) at 100V until the dye front reached a distance of approximately 1 inch from the end of the gel. The gel was stained in 1.0 μg/ ml ethidium bromide for 5-10 minutes, and then destained for 30 minutes to 1 hour in dH$_2$O. The gels were next placed on a UV Transilluminator for visualization. Documentation was performed using the PhotoDoc-It Imaging System (UVP, LLC) to photograph each gel.

Making of Infectious Doses

RB51-QAE colonies from the electroporation experiment that were proven to contain pQAE were spread on three BA-AMP plates and incubated at 37ºC in a 5% CO_2 atmosphere for approximately 72 hours. The cells were harvested from each plate using 2 ml of sterile broth per plate and the cells from all plates were combined in a single tube. A tube containing 12 ml sterile broth was then inoculated with 50 μl of the harvested cells and the optical density (OD) was determined (at 600nm). The desired absorbance reading of 0.150 was obtained and is indicative of a $1x10^9$ dilution. Using this information, two tubes were prepared; the first with 6 ml broth and 2.5 ml culture for a dilution of $1x10^{11}$, and the second with 24 ml broth and 100 μl culture for a dilution of $1x10^9$. These dilutions were divided into 1 ml portions and placed in individual tubes, which would serve as the infectious doses for this experiment. The tubes were flash frozen and placed in a -80ºC freezer until needed. Plate counts were performed to ensure the approximate cell count for one tube from each of the dilution groups.   The results of these plate counts confirmed an accurate CFU/ ml for each of the dilutions tested.

Animals

Twenty *Brucella* negative goats were divided into 2 groups of 10. Group 1 was inoculated conjunctivally with commercially available RB51 vaccine ($1x10^{10}$ colony forming units), group 2 with the RB51-QAE vaccine ($1x10^{10}$ colony forming units). Blood samples were collected from each animal prior to inoculation and on the day of necropsy. Initial blood samples were collected using Precision Glide Vacutainer Blood Collection Needles (Becton Dickinson and Co.) in association with 10 ml BD red-top Vacutainer Serum tubes (Becton Dickinson and Co.). The blood samples were allowed

to clot overnight then centrifuged for separation. The serum was drawn off, subjected to
a Brucellosis card test, then stored for later use at -20°C. Blood collected at necropsy
was obtained through exsanguination.

From each of the groups, two goats were euthanized at 7, 14, 21, 28, and 42 days
post-vaccination using captive-bolt and exsanguination methods. Tissue samples
obtained at necropsy included the parotid lymph node, prescapular lymph node, liver
portions, spleen portions, internal iliac lymph node, and the supramammary and inguinal
lymph nodes of female and male goats, respectively. The individual tissues were
homogenized in 20 ml PBS, and 100µl were spread onto *Brucella* selective media (Oxoid
Ltd., Basingstoke, Hampshire, England) (Farrell 1974) then allowed incubate at 37°C for
a period of 2 weeks.

Bacteriological Examination

Resultant colonies were confirmed as *Brucella* or non-*Brucella* by the
morphological appearance of each colony and the activity of the colonies in question
when submitted to oxidase, catalase, and urease tests. Suspect RB51-QAE colonies were
also subjected to plasmid isolation and PCR targeting region E to ensure the presence of
region E in these cells. The confirmed *Brucella* colonies were counted and recorded in
the form of a growth chart comparing the time of sacrifice post-immunization and the log
CFU per organ for each of the vaccines tested.

A series of 10 biochemical tests were also performed to compare the results of *B.*
abortus 2308, RB51, and RB51-QAE. *B. abortus* 2308 was included because it is the
smooth parental strain of RB51. These tests included a Gram stain, serum agglutination,

an acriflavin test, oxidase, catalase, urease, H₂S, and TSI tests as well as dye sensitivity tests with Thionin and Basic Fuchsin.

Serum agglutination was performed to determine a rough or smooth phenotype of RB51 and RB51-QAE. This test was performed by placing a drop of commercially available antisera against *Brucella* OPS (Fisher Scientifics) onto a clean glass slide and mixing with it a suspect colony of RB51 from a Brucella Selective blood agar plate. The same procedure was performed for suspect colonies of RB51-QAE. The results were read based on the occurrence or absence of agglutination observed on the slide. Smooth brucellae will cause agglutination, whereas rough brucellae will not.

Gram negative organisms with a rough phenotype will agglutinate when mixed with acriflavin. This test was used as another method of determining the rough or smooth characteristics of RB51 and RB51-QAE. For this test, a fresh solution of 10mg acriflavin in 10 ml of sterile dH2O was made. A small volume of this solution was placed onto a clean glass slide, then mixed into suspension with a colony of either RB51 or RB51-QAE from an agar plate. The suspensions were observed for agglutination and the results documented.

RB51 and RB51-QAE were also tested for the ability to produce cytochrome oxidase, which reduces molecular oxygen, using the oxidase test. An oxidase reagent ampule (Becton Dickenson and Co., Cockeysville, MD) was prepared by breaking the ampule and mixing the contents within. Using a sterile cotton swab, a colony of either RB51 or RB51-QAE was selected from an agar plate. The oxidase reagent was dropped onto each swab and observed for 30 seconds. The results were read by the absence or appearance of a violet color on the swab within 30 seconds.

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Catalase is an enzyme capable of converting hydrogen peroxide into oxygen and H_2O. To test for the presence of this enzyme in RB51 and RB51-QAE, a loop of each organism was chosen from an agar plate and smeared onto a clean glass slide. One to two drops of a commercial catalase reagent (Becton Dickenson and Co.) was added to each slide and mixed with the suspect colonies of each strain. The test was read by the formation or absence of oxygen bubbles forming within the mixture.

To test for the ability of RB51 and RB51-QAE to produce urease, which breaks down urea into ammonia, CO_2 and H_2O, a urease test was performed. Urease slants (Remel, Inc.) containing a pH indicator were streaked with an isolated colony of either RB51 or RB51-QAE. The urease slants were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. The results were read by the presence or absence of a pink color produced by the pH indicator, which will change colors in a basic environment.

A Hydrogen Sulfide (H_2S) test was performed to test the ability of each strain tested to produce H_2S from amino acids containing sulfur. Triple sugar iron (TSI) media (Remel, Inc.) was inoculated with either RB51 or RB51-QAE. A commercially available lead acetate strip (Sigma-Fluka, Inc.) was secured between the cap and inner wall of the media tube. The tubes were incubated overnight at a temperature of 37°C in a 5% CO_2 atmosphere. The results were read by observing the development or absence of a black color on the lead acetate strip. Using the same TSI media inoculated with either RB51 or RB51-QAE, a TSI test was performed. The results were read at the same time as the H_2S test by observing the media for changes in color and the production of gas bubbles.

Dye sensitivity tests were performed for each strain using Thionin and Basic Fuchsin. Plates were made using agar containing either Thionin or Basic Fuchsin (Hardy

Diagnostics) then streaked with suspected colonies from RB51 or RB51-QAE that had been suspended in a sterile saline solution. A positive control plate was also prepared. Each plate was incubated at 37°C in a 5% CO_2 atmosphere for a period of 3-4 days to allow for adequate growth. The results were determined by the presence or absence of growth in the presence of Thionin and Basic Fuchsin.

Serological Examination

A blood sample from each of the test animals was taken prior to infection and at the time of sacrifice. These samples were spun down and the serum extracted. The Brucellosis Card Test (Obtained from USDA, APHIS, manufactured by Becton Dickenson and Co.) was performed for each of the pre-bleed samples as well as for each of the post-inoculation samples. A 30µl volume of Buffered Brucella Antigen (BBA) (Becton Dickinson and Co.) was placed onto a Brewer Diagnostic Card (Becton Dickinson and Co.) with a 30µl sample of serum from each goat. A sterile toothpick was used to mix each serum sample with the BBA until a uniform suspension was achieved. The samples were then rocked for a period of 4 minutes and observed for agglutination.

The serum from each goat was also used to perform western blots on all pre-bleed and post-inoculation samples for the purpose of monitoring the antibody immune response for each animal. The blots included lysates of both RB51 and RB51-E, as well as several other strains of *Brucella,* both rough and smooth.

Cell lysates of *B. abortus* 2308, *B. abortus* RB51, *B. abortus* RB51-QAE, *B. melitensis* 16M, *B. suis* VTRS-1, *Y. enterocolitica* 0:9. and *Y. enterocolitica* 0:8 were prepared for western immunoblots through the method of sonication. For sonication, each species of bacteria were grown on selective media plates. Each culture was

incubated for 2-3 days at 37°C in a 5% CO_2 atmosphere. The bacteria were harvested using 2 ml of sterile PBS (Sigma Chemical Company) for each plate. Each volume of 2 ml was sonicated using a Heat Systems-Ultrasonics W-385 Sonicator (Farmingdale, NY) for 8 minutes with a 50% duty at a 1-second pulse and a 4.0 to 4.5 output. A loop of the resultant lysates were spread onto one half of a selective media plate and the rest boiled for 10 minutes. A loop of the sonicated and boiled lysates were spread on the second half of the selective media plate and allowed to incubate for 2-3 days to confirm that the *Brucella* cell lysates are no longer viable. To prepare the lysates for western immunoblotting, a 150μl volume of lysate was mixed with a 150μl volume of Laem mli Sample Buffer (BioRad Laboratories) prepared with a 0.1 volume of 2-mercaptoethanol and boiled for an additional 10 minutes.

Coomassie staining was used to determine the ideal volume of each lysate to be used in polyacrylimide gel electrophoresis (SDS-PAGE). Based on these results, volumes between 3 and 5μl of each lysate was loaded onto a precast 12% Tris-HCl Ready Gel (BioRad Laboratories) with a Kaleidescope Prestained Standard (BioRad Laboratories) size standard marker. The gels were run in a Criterion Precast Gel System (BioRad Laboratories) at 150V for a period of 1 hour and 15 minutes to 1.5 hours until the dye front reached the bottom of the gel.

Proteins were transferred to a nitrocellulose membrane (Osmotics, Livermore, CA) at 100V for 1 hour at 4°C. The membrane was blocked in a 5% solution of Blotting Grade Blocker Nonfat Milk (BioRad Laboratories) in Tris-Buffered Saline (0.5M NaCl, 20mM Tris) (TBS), incubated for 1 hour on a rocker at room temperature, washed with TBS-Tween (TBS, Tween-20) 5 times and once with TBS. Each individual blot was

incubated overnight in a 1:40 dilution of test serum on a shaker at room temperature, and washed again as before. The immunoblots were immersed a 1:800 dilution of rabbit anti-goat IgG horseradish peroxidase (Sigma-Aldrich Co., St. Louis, MO) on a shaker in room temperature for 45 minutes. Blots were developed in a TBS-methanol-3% hydrogen peroxide solution using 4-chloro-1 napthol tablets (Sigma-Aldrich Co.) for visualization, stopped in dH_2O, and air dried.

Histopathological Analysis

Histopathology was performed by Dr. Eric Snook at the School of Veterinary Medicine, Louisiana State University. Tissue samples from necropsy were fixed in 10% neutral buffered formalin and embedded in paraffin. Each slide was stained using hematoxylin and eosin then examined for pathology.

Results

Creation of B. abortus RB51-QAE

The resultant colonies that grew on the BA-Amp plates following electroporation were subjected to further testing in order to ensure that the surviving colonies were *Brucella* spp. and carried the pQAE DNA with the region E gene (Figure 1 A & B). All colonies tested catalase, oxidase, and urease positive, confirming the colonies as *Brucella* spp. A plasmid isolation miniprep was performed on the suspect RB51-QAE colonies and further tests such as PCR and restriction enzyme digestion, both targeting region E of the plasmid DNA, were used for confirmation.   Sequencing information obtained for pQAE was analyzed and confirmed the presence of the region E gene sequence in pQAE.

The resultant plasmid DNA from the miniprep procedure was run on an agarose gel with a size standard and a positive and negative control, stained with ethidium

(A) Region E Gene Sequence

GAATTGGCGACCTGACTGAGGATAGCGGCACAAATACGGCTGCAGTCAGCGGCAATACCATTCGCGCCC
AGACGATCGTGAATGATAGTTCTAATACCCTTGAAGGAAAACTGTCGGACGACTATGCGTCATCAACGTT
GGGTACCTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGCAATCC
TGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGAGGGCATAATAGGAA
CCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAGTCTGCAAGAAAATACTATTGATG
CTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGCCATCGACAAGGAAGGCGATCCGACTTTCCAAG
GCTCGGTCCTTATTACCAATCTGCAGCAGAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGG
GTATATTGGCGCAAGCGACAGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGT
AGCGGAAAACATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCAT
CAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTCAACGATATCGC
ATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGCCAATAACCAGGCAAACATCGT
TACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGTCTATTGGTGTGGAGGGTACACCGACGATTGGT
GCGGTTGTTGATGCCGTCGAAGGAGGTTCGGTTGTCCTCGCGGATAATGCCGTGACGTCTCAGGCGGTAG
GTAACAACACATCCGCCGCAATCTTGAAGGATGATGACTCCGCAGTCGGCTTTGATGCAACAGCTGCCCT
TGCTAACCATCAGATTAATCTGTTTTCTGATATTGCGGCGACCACGCAAAATGGCTCAGTGGTTGCCATTG
TCGGTAAAGCGAGAGACAGTATCTTCGATGAAGGGACCGTTGATGTTTCGGGGAACAAGATTTCCGCACT
GGCGTTTGGCAACAGCGCCAGCCAGCAGTTGGCCCTGGACGCCAATAACCTAACCGCTGGGGATAGCAC
TGGGGTTGCTGACGGGCGGGCCGAACGACGAGACTACCCATGATAGCGGCCTTCGGGCTAAGGCAGGTGC
TATGCTTACCAGCCTACAGGCAAACTATAGCAGTGATATTTCCGCCAACAATGCGGCGTCGGTGGTTGGT
GTCTACGGCGACAACAAGGTTGGTAGCGACATATCTGGAGCCAAATTAACCGTCGAGAACAACACGCAG
CAGGCGACTGCGATTGGTAGCGACGCTACTAATCTGCTTGGACAAGTGCATTATGAGGACGGCAAGGCG
GATCATGTAGCCGGACTTGGCGGAAACAGTGTCGCCGGTTCGGCCGGTATTGCCAACGTTCAGGTGGGTG
ATGCTGGATCGTCGGTCATTGCTTCGTTGACCGATGCGGTCGCAGGATTCCCAGGAATCAGCAGTAGGGC
TCCCATAACGCTACCGTTTTTTAATGCCAACGTTAAACAGGAGGAATCCAGCTTTTCGGTAACCGACAAT
GTGCAGAGCGCCTCAGCCAGCGGCACCCAGAGCCGCAATGAACTGGTGGTCGAGAGCAATTCCGTCACT
GCCAATATTGGAACTGGTGCTCAAGAGCATCCCACTTCATCAAATACCGGTCTTGACGGAGCGTATGTAC
GCGATAACGAAGATAGTTTCCATACCATTCATCAGCCTATGATCATGGCGGCCTATGGCCTTATCAACGA
TCAGTCGATTGGGGGGAGAGTCAATGCCCTAGAACCTGTCTGCATTCAAGGATTCCCTTTTGTACGAAAT
TCTGATTCAAGGTTGTTAAAGGAGAACAGCCGTGAG

FIGURE 1

(A) Region E gene sequence with ORF-944F and ORF-944R primer sequences in red.

(B) Diagram of the pBBR1MCS-4 plasmid used to create pQAE.

(B)
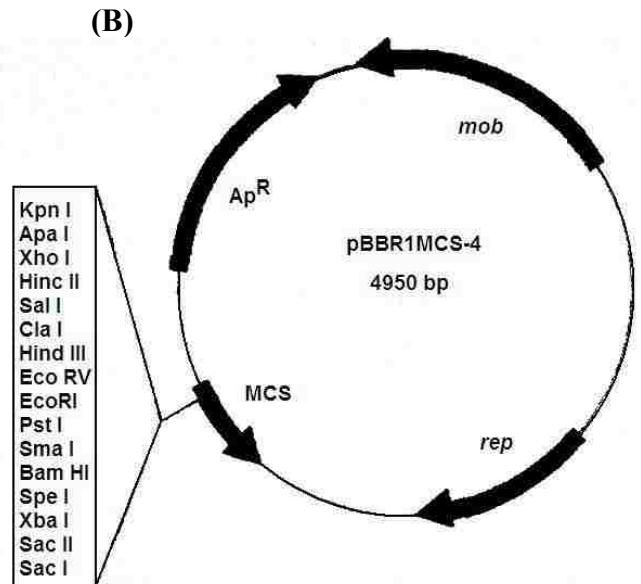
bromide, and analyzed under UV light. A band of approximately 4950 bp in length was observed for all of the plasmid DNA tested, suggesting that the colonies in question carried the 4950 bp pQAE (data not shown).

A PCR experiment was set up and run with primers (Orf944F and Orf944R) targeting the region E portion of pQAE miniprep isolated from the suspect RB51-QAE colonies. The resultant amplified DNA was run on a 1% agarose gel with a supercoiled DNA molecular weight ladder and controls, stained, and analyzed under UV light. A band of approximately 2000 bp was observed in each of the test sample lanes, confirming the presence of region E in the plasmid DNA isolated from the suspect RB51-QAE colonies (Figure 2).

For further confirmation, a restriction enzyme digest using EcoRV, targeting region E, was also performed on the plasmid DNA from the colonies in question. The digests were run on a gel with a size standard, controls, and the intact plasmid DNA of each digested sample. The resultant gel produced bands of approximately 4950 bp in length corresponding to the intact DNA, and bands of approximately 2000bp in length corresponding to the lanes of digested DNA (data not shown). Therefore, the results of this digest further confirmed the presence of region E in the plasmid DNA isolated from the suspect RB51-QAE colonies.

Due to the results of the plasmid isolation and subsequent sequencing, PCR, and restriction enzyme digest performed on the RB51-QAE colonies in question, it is confirmed that the electroporation procedure was successful and that the resultant RB51-QAE colonies carried the QAE plasmid containing the region E gene.
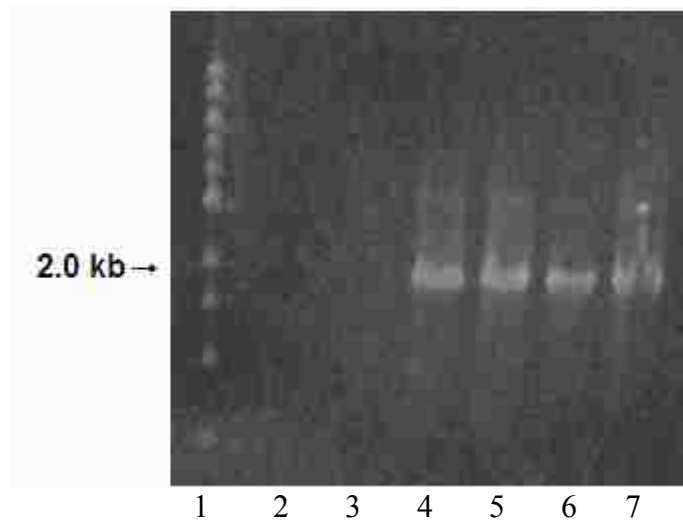
Figure 2.

Agarose gel electrophoresis of plasmid minipreps performed on 3 suspected RB51-QAE colonies amplified by PCR using the ORF-944F and ORF-944R primers and run by agarose gel electrophoresis. The bands are approximately 2000bp in size, according to the standard molecular weight marker shown.

The lane order is as follows:
    Lane 1 – 1kb Molecular Weight Ladder
    Lane 2 – Negative control group of dH$_2$O
    Lane 3 – PCR products from *B. abortus* RB51
    Lane 4 - PCR products from miniprep DNA isolated from RB51-QAE suspect 1
    Lane 5 - PCR products from miniprep DNA isolated from RB51-QAE suspect 2
    Lane 6 - PCR products from miniprep DNA isolated from RB51-QAE suspect 3
    Lane 7 – Positive control of PCR products from pQAE DNA

Bacteriological Examination

In order to better characterize RB51-E, 10 general biochemical tests, including dye sensitivities were performed for RB51-QAE, the standard RB51 vaccine strain, and *B. abortus* 2308. The results of these tests for RB51 and 2308 matched the typical results seen for RB51 and 2308. The results produced by RB51-QAE were identical to those seen for the standard RB51 vaccine strain (Table 1).

To evaluate the colonization capabilities of RB51-QAE in the caprine host, twenty non-pregnant goats were conjunctivally inoculated with 1×10^{10} colony forming units (CFU) of either RB51-QAE or RB51. Following the necropsy of each animal at 7, 14, 21, 28, and 42 days post inoculation, each of the 6 tissues collected per animal were homogenized and a portion of each tissue was plated onto a BS-BA plate as well as a BA-Amp plate. The resultant colonies from each animal at each time of sacrifice were counted and recorded. The overall culture results showed that the tissues from animals inoculated with RB51-QAE yielded a significantly higher bacterial titer at 14, 21, and 28 days post-inoculation than the tissues from animals inoculated with RB51. RB51-QAE was also shown to survive longer in the host tissues than standard RB51 (Figure 3) (Table 2). It should be noted that the results obtained for 14 days post-inoculation for the RB51 group are not typical when compared to previous studies examining the survival of RB51 in the caprine host. In most instances, RB51 is capable of surviving up to 21 days post-inoculation. The absence of RB51 in the 14 day post-inoculation group could be due to errors in vaccine administration or deficiencies within the animals tested.

Only one confirmed *Brucella* colony was detected for either group at 42 days post inoculation (Table 2). This CFU was observed for an RB51 animal, but after further

59

Table 1.

Results of ten biochemical tests performed on 2308, RB51, and RB51-QAE. The results for 2308 and RB51 were expected and consistent with previous findings. RB51-QAE produced results similar to RB51, indicating that the addition of the pQAE plasmid did not make RB51-QAE biochemically dissimilar to the parental strain.

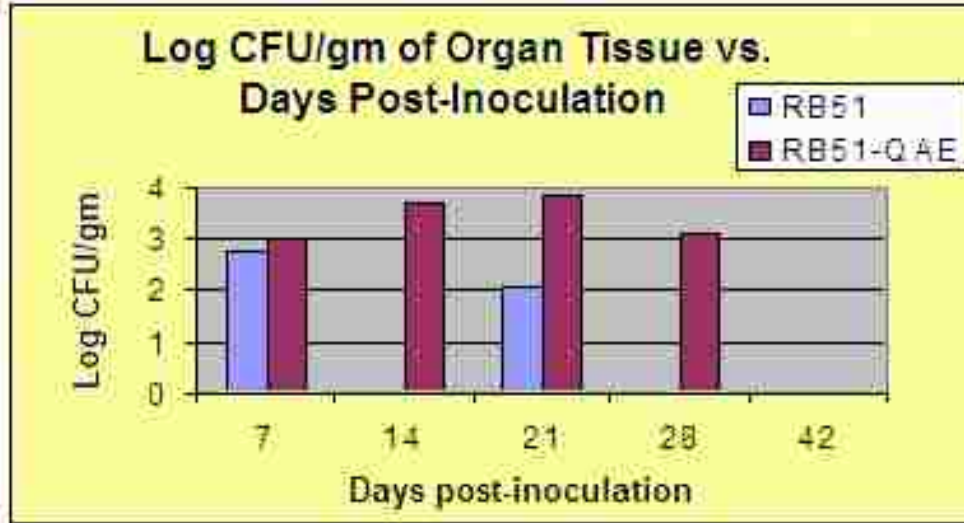| Test	R or S	Gram Stain	Serum Agglutination	Acriflavin	Oxidase	Catalase	Urease	H2S	TSI	Thionin	Basic Fuschin
2308	Smooth	neg	+	–	+	+	+	+	–	–	+
RB51	Rough	neg	–	+	+	+	+	+	–	–	+
RB51-QAE	Rough	neg	–	+	+	+	+	+	–	–	+

Figure 3. Colonization of male and non-pregnant female goats with RB51 and RB51-QAE in average CFU/gm of tissue from parotid and prescapular lymph nodes taken at 7, 14, 21, 28, and 42 days post inoculation. Significance considered at p<0.05.

Table 2. Colonization of goats with *B. abortus* RB51 and *B. abortus* RB51-QAE in Parotid Lymph Nodes, Prescapular Lymph nodes, Liver, and Spleen

	Animal #	Received	Card Test	Par LN	CFU/g	PreS LN	CFU/g	Liv/Spl	CFU/g
7 DPI	O-26	RB51	negative	37	3.72E+03	No Growth	–	2	4.22E+01
	26	RB51	negative	4	8.19E+02	No Growth	–	1	2.36E+01
	O-20	RB51-QAE	negative	40	4.58E+03	No Brucella	–	1	2.00E+01
	Y-32	RB51-QAE	negative	62	2.46E+03	No Growth	–	1	1.98E+01
14 DPI	O-30	RB51	negative	No Growth	–	No Growth	–	No Growth	–
	Y-28	RB51	negative	No Growth	–	No Growth	–	No Growth	–
	Y-08	RB51-QAE	negative	250	3.55E+04	1	6.43E+01	No Growth	–
	O-07	RB51-QAE	negative	4	7.05E+02	No Growth	–	1	2.20E+01
21 DPI	O-14	RB51	negative	No Growth	–	No Growth	–	No Growth	–
	Y-19	RB51	negative	4	9.12E+02	No Growth	–	No Growth	–
	O-29	RB51-QAE	negative	315	2.36E+04	1	5.03E+01	1	1.97E+01
	Y-23	RB51-QAE	negative	200	3.11E+04	No Growth	–	6	2.31E+01
28 DPI	Y-06	RB51	negative	No Growth	–	No Growth	–	No Growth	–
	O-08	RB51	negative	No Growth	–	No Growth	–	No Brucella	–
	Y-07	RB51-QAE	negative	8	2.77E+03	No Brucella	–	No Brucella	–
	G-54	RB51-QAE	negative	39	6.41E+03	7	5.09E+02	6	2.33E+02
42 DPI	O-24	RB51	negative	1	1.09E+02	No Brucella	–	No Brucella	–
	PD3	RB51	negative	No Brucella	–	No Growth	–	No Brucella	–
	O-23	RB51-QAE	negative	No Brucella	–	No Brucella	–	No Brucella	–
	Y-27	RB51-QAE	negative	No Growth	–	No Growth	–	No Growth	–

testing by plasmid isolation and PCR analysis, it was found to be an RB51-QAE colony. It was assumed that this colony was the result of a contamination during necropsy.

Serological Examination

Each of the animals used for this study were subjected to pre-inoculation and post-inoculation serum testing. A pre-inoculation serum sample and a post-inoculation serum sample, taken at the time of sacrifice, were tested for each animal by a standard brucellosis card test. Each of the animals tested negative before and after inoculation for *Brucella*-specific smooth antibodies. The pre and post inoculation samples were used to run a set of western blots for each animal. The western blots contained the lysates of *B. melitensis* 16M, *B. suis* VTRS-1, *B. abortus* 2308, RB51, RB51-QAE, *B. ovis*, and *B. canis*. The results of RB51-QAE inoculated goats on the western immunoblot assay yielded an equal or greater humoral immune response in the goats tested as compared to blots run for RB51 goats during the same time points (Figure 4).

Histopathology

There appeared to be no definitive histological pattern in the liver and spleen with respect to the time or type of infection in any animal. At day 28, the RB51 group subjectively appeared to have an increased follicular activity as compared to RB51-QAE in some lymph nodes. At day 42 the RB51-QAE group had a subjectively increased follicular activity over RB51 (data not shown).

Discussion

In 2007, Perry hypothesized that addition of the Region E sequence via the pQAE plasmid to *B. abortus* 2308 would increase virulence in the caprine model and that the absence of Region E from *B. melitensis* 16M would cause attenuation in the caprine

21 days post-inoculation   28 days post-inoculation

1 2 3 4 5 6 7
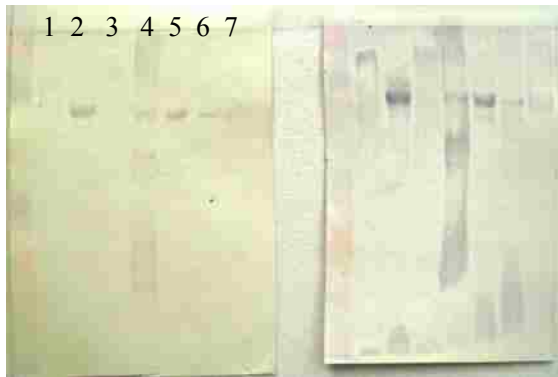
42 days post-inoculation

Figure 4.

Characteristic western immunoblots showing an increase in humoral response in goats inoculated with RB51-QAE. These results are from representative goats sacrificed at 21, 28, and 42 days post-inoculation. The first blot in each group shows the results of a pre-inoculation sample and the second shows the results of the post-inoculation sample taken during necropsy on the days specified.

The order of lysates, as follows, is numbered on the first blot of this figure and the same order remains throughout:

1. *B. melitensis* 16M   5. *B. abortus* RB51
2. *B. abortus* 2308   6. *B. canis*
3. *B. suis* VTRS-1   7. *B. ovis*
4. *B. abortus* RB51-QAE

model described by Elzer et al. in 2002. It was demonstrated that the addition of Region E to *B. abortus* 2308 resulted in a significant increase in colonization and pathogenicity in goats, when conjunctivally inoculated. The *B. melitensis* 16M Region E deletion mutant (16MΔE) did not cause decreased colonization when compared to the parent strain, but did result in a decrease in pathogenesis in the caprine host (Perry 2007). Further investigation of the Region E protein as a putative hemagglutinin and host specificity factor was merited.

This study uses the caprine model to test the effects of the Region E gene addition to *B. abortus* RB51 (RB51-QAE) . Furthermore, the characterization of the Region E protein as a putative hemagglutinin, cell surface protein, host specificity factor, and immunogenic determinant was explored. It was hypothesized that addition of the Region E protein, via pQAE, would increase virulence of RB51 in the caprine model described by Elzer et al. in 2002. It was also proposed that the Region E protein was a cell-surface hemagglutinin and host specificity factor capable of inducing anti-Region E protein antibodies in the caprine host. Both the parental strain of RB51 and the RB51 region E variant (RB51-QAE) were examined in the caprine host for effects on colonization using both male and non-pregnant female goats. The hemagglutinating abilities of the region E protein were evaluated using RB51 and RB51-QAE in a hemagglutination assay examining cell harvest, broth culture, and supernatant samples using both goat and cow erythrocytes. Absorption assays were also performed using serum from goats infected with comparison strains of *Brucella* with and without the presence of the Region E gene sequence (specifically, 16M and 16MΔE, 2308 and 2308-QAE, RB51 and RB51-QAE).

The RB51-QAE mutant was examined using ten biochemical tests (described previously) and it was demonstrated that the addition of pQAE did not make the organism biochemically different from the parental strain. When subjected to a Rose Bengal card test, serum from neither experimental group provided positive test results. This indicates that the addition of pQAE to *B. abortus* RB51 did not result in converting the strain from rough to smooth. Furthermore, a noticeable increase in humoral detection by western immunoblots was observed when compared to the parental RB51 strain, suggesting that RB51-QAE has an increased immunogenicity when compared to the parental strain.

The addition of pQAE provided RB51-QAE with specific attributes that may have enabled the organism to increase its colonization in a caprine host. At day 7 post-inoculation, both RB51 and RB51-QAE successfully colonized the parotid lymph nodes and were detected in the liver and spleen. This outcome was typical of an RB51 colonization in the caprine model. At day 14 post-inoculation, only RB51-QAE was cultured from necropsy. None of the RB51-inoculated animals produced Brucella colonies from any of the tissue samples tested. This may be due to an error in sample collection, sample preparation, or bacteriologic technique. Additionally, this result may also be attributed to inoculation errors or physiological issues involving the animal itself. Typically, RB51 is able to colonize tissues of the caprine host for approximately 21 days post-inoculation, which further supports our day 14 post-inoculation results as erroneous (Adone et al. 2005, Neilson et al. 2004). As shown by the day 21 post-inoculation animals, RB51 continued to colonize the parotid lymph nodes in the animals tested, indicating the erroneous nature of the day 14 post-inoculation findings. In this same

group, the RB51-QAE goats sacrificed at 21 days post-inoculation were identified in the parotid lymph nodes, the prescapular lymph node, and in liver and spleen tissues. In addition, only RB51-QAE was cultured from the day 28 post-inoculation animals, supporting the proposal that RB51-QAE is able to survive for a longer period in the caprine model than the parental strain. On day 42 post-inoculation, no significant culture growth was detected from any of the animals or tissues tested. A single colony was detected and confirmed as RB51-QAE, but this result is not significant.

The results of the tissue culture assays show that RB51-QAE is able to colonize the host more effectively than the parental strain RB51. The results of this experiment as a whole merit further investigation into the function of RB51-QAE as a vaccine candidate. Future studies to determine the pathogenicity and, subsequently, vaccine efficacy in goats and other primary host and reservoir animals are currently in planning. Because smooth vaccines interfere with diagnostic tests and are an obstacle to eradication efforts, a rough vaccine applicable in goats and would be a useful tool in reducing the disease in endemic countries.

CHAPTER TWO

CHARACTERIZATION OF A PUTATIVE HEMAGGLUTININ OF *BRUCELLA MELITENSIS* EXPRESSED BY THE REGION E GENE

Introduction

Determining the functional characteristics of the protein expressed by the region E gene is important in evaluating its role in the increased colonization capabilities of RB51-QAE in the caprine host. Strains of *B. abortus* lack the region E gene and are not as effective in colonizing goats as strains of *B. melitensis*, which naturally express the region E gene. The region E protein was shown to increase colonization of both *B. abortus* 2308-QAE and RB51-QAE in goats, suggesting that the addition of region E may confer host-specificity to strains of *B. abortus* in the caprine host. The protein expressed by region E is referred to as a putative hemagglutinin and cell wall surface protein when examined through a BLAST search.

In evaluating the potential of RB51-QAE as an improved vaccine candidate, it is important to determine if the protein is expressed by RB51-QAE and, if expressed, the functional aspects of the region E protein in the caprine host. Two-dimensional gels were created and mass spectrometric analysis was used to determine expression of the region E protein in RB51-QAE and a strain of *E. coli* DH5α that has been made to express the region E gene (DH5α-TOPOE), but the protein was not detected in the gels. Hemagglutination assays were prepared with bacterial strains expressing pQAE to evaluate the hemagglutinating capabilities of region E protein. The immunogenic characteristics of region E were assessed using western blot absorption analysis using serum from goats inoculated with strains of *Brucella* expressing the region E gene.

Materials and Methods

Preliminary Characterization

The region E gene was analyzed using the NCBI Basic Local Alignment Search Tool (BLAST) and the *B. melitensis* genome surrounding region E was examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) online database.

Bacterial Preparations for Hemagglutination Assay

The strains of bacteria studied in the hemagglutination assay were chosen based on the presence or absence of the region E protein in the genome and tested against mutant strains that either contained a plasmid expressing the region E gene, or a deletion mutant that is devoid of the region E gene sequence. The bacterial strains used are as follows: *B. abortus* RB51 & *B. abortus* RB51-QAE and *E. coli* DH5α & *E. coli* DH5α-TOPOE. Previously stored cell lysates for all of the strains tested were subjected to hemagglutination assays using goat erythrocytes. Both strains of *E. coli* DH5α were grown previously and stored in glycerol stocks at -80°C. DH5α-TOPOE expresses the TOPOE plasmid, which is a high-expression vector that contains a copy of the region E gene as well as a kanamycin resistance gene for selection. To confirm the presence of the region E gene in the TOPOE plasmid, the plasmid was sequenced using BioMMED (Baton Rouge). Both *B.abortus* RB51 and RB51-QAE lawns were grown as previously described on selective SBA plates, with RB51-QAE being grown on plates containing ampicillin at a concentration of 1μl/ ml media. Two series of SBA plates were prepared with one set containing bovine blood and the second containing caprine blood. Both RB51 and RB51-QAE were grown on both sets of blood SBA plates and harvested in 2 ml of sterile PBS (Sigma Chemical). Both *E. coli* DH5α and *E. coli* DH5α-TOPOE were

grown on LB agar media and similarly harvested. The LB agar plates used to grow

DH5α-TOPOE were prepared with a 1μl/ ml media concentration of kanamycin for

selective purposes. All cell suspensions were analyzed by spectrophotometer at an

OD_{600nm} reading of 0.75 to equalize cellular concentrations of each experimental group.

Each sample was then subjected to hemagglutination assays.

Broth cultures were also prepared for each of the RB51 and DH5α strains in 2 ml

of broth in a 15 ml centrifuge tube. *B. abortus* bacteria were grown in brucella broth

(Becton Dickinson and Co.). Ampicillin was added to the broth culture containing

RB51-QAE at a concentration of 100 μg/ ml of broth. Control tubes were also set up

with one control brucella broth culture containing no specimen and a brucella broth

culture containing ampicillin inoculated with RB51. Broth cultures for *E. coli* were

prepared in LB broth. Kanamycin was added to the broth culture containing DH5α-

TOPOE. A control tube was prepared with LB broth and no added inoculate. A second

control was also prepared using LB broth containing kanamycin at a concentration of

45μg/ ml of broth and inoculated with DH5α. Each culture was placed in a water bath set

to 37°C with moderate agitation. The *E. coli* cultures were allowed to incubate

overnight, and the *B. abortus* cultures were allowed to incubate for a period of 3 days.

Following incubation, a 500ul portion of culture was separated and centrifuged. The

supernatant was separated and subjected to a hemagglutination assay. The remaining

broth culture was subjected to a hemagglutination assay as a whole.

Erythrocyte Preparation

Erythrocytes from both bovine and caprine sources were used for the

hemagglutination assays to assess the host-specificity of the putative hemagglutinin. A

blood sample was collected using a 10 ml BD Vacutainer tube (Becton Dickinson and Co.) containing EDTA as an anticoagulant. The sample was mixed and aliquoted into 500μl portions in sterile 1.5 ml micro centrifuge tubes. The contents of each tube were centrifuged at 3000rpm for 3 minutes, the plasma was drawn off, the erythrocyes were sspended in 500μl of sterile PBS. This rinsing step was repeated 4 additional times and the final erythrocyte suspension was stored for no more than 3 days at 20°C. Both caprine and bovine erythrocytes were prepared and stored in this manner and used to make a 2% solution for each of the hemagglutination assays.

Hemagglutination Assay Technique

Hemagglutination assays were prepared in 96 Well "U" Bottom Plates (Corning Incorporated, Corning, NY) containing 100μl sterile PBS per well with (Sigma Chemical) 200μl of each cell suspension or culture sample in the first well of the series. Two fold dilutions were made by adding 100μl of the initial sample into 100μl of PBS in the next well and 100μl of either bovine or caprine erythrocytes were added to each well. A control of PBS was also included. The samples were allowed to incubate overnight.

A hemagglutination assay using only the supernatant from each broth culture was also prepared to assess localization of hemagglutinating factors as either cell associated or secreted using similar techniques. The supernatant samples were not adjusted according to OD_{600nm} calculations, as readings were identical, suggesting that a comparable amount of supernatant proteins were already present in each sample.

Visual Analysis

Plates were read visually according to the presence of a button or matte in the well, the size and color of each button present, and the color and clarity of the PBS

suspension in each well. Because the bacterial cells settled to the bottom of the well, each plate was read from the bottom.

Absorption Assay

Absorption assays were performed to detect host antibodies to the region E protein by way of western immunoblot for the purpose of further characterizing the region E protein as it is expressed *in vivo*. Samples were obtained from goats previously infected with *B. abortus* 2308-QAE, *B. melitensis* 16M, and *B. abortus* RB51-QAE. The serum from goats infected with strains of 2308-QAE and 16M were from a previous experiment performed in this lab and the serum sample from the RB51-QAE goat was from the colonization experiment previously described.

An amount of 1 ml of each serum sample was combined with 1 ml of 2308 (for 2308-QAE), RB51 (for RB51-QAE) and 16MΔE (for 16M) lysates in a sterile microcentrifuge tube. The tubes were then placed on a rocker for 2 hours to allow absorption of *Brucella*-specific antibodies. The tubes were removed from the rocker, centrifuged at full speed for 5 minutes to pellet cells, then the remaining serum was drawn off for western blot analysis.

The western blot analysis to detect antibody to the region E protein was performed as previously described and the lane order was as follows: a size standard marker, RB51, RB51-QAE, 2308, 2308-QAE, 16MΔE, and 16M. The western blots were analyzed visually following development.

Membrane Isolation

Two dimensional gels were run for both *B. abortus* RB51 and RB51-QAE as well as *E. coli* DH5-α and DH5-α-TOPOE. Whole cell lysates were used for all species and

purified membrane components were obtained for DH5-α and DH5-α-TOPOE. To

isolate membrane components for DH5-α and DH5-α-TOPOE, a 5×10^8 CFU/ ml culture

was grown overnight at 37°C. Cells were pelleted and resuspended in .75M sucrose-

10mM tris. Lysozyme and EDTA were added to the suspension and the cells were

pelleted. The supernatant was drawn off and ultracentrifuged at 60,000 rpm to isolate

membrane portions.

2D Gel Analysis

Protein samples were cleaned using the Ready Prep 2D Cleanup Kit (BioRad

Laboratories). One hundred μl of each lysate was mixed with 300μl precipitating agent 1

(BioRad Laboratories), then incubated on ice for 15 minutes. Three hundred μl of

precipitating agent 2 (BioRad Laboratories) was added and the tubes were centrifuged at

14,000 rpm for 5 minutes for pellet formation. The supernatant was removed, 40μl of

wash reagent 1 (BioRad Laboratories) was added to the pellet, which was centrifuged at

14,000 rpm for 5 minutes. Next, 25μl of Ready Prep proteomic grade water (BioRad

Laboratories) was used to resuspend the pellet. One milliliter of pre-chilled wash reagent

2 (BioRad Laboratories) was added with 5μl of wash 2 additive (BioRad Laboratories)

then vortexed for 1 minute. Each tube was incubated on ice for 30 minutes, with a 30

second agitation every 10 minutes during incubation. The tubes were centrifuged at

14,000 rpm for 5 minutes to form a pellet. The pellet was allowed to air dry at room

temperature then resuspended using 2D Rehydration buffer (BioRad Laboratories). The

samples were centrifuged at 14,000 rpm for 5 minutes. The supernatant was drawn off

and run on Immobilized pH Gradient (IPG) strips (BioRad Laboratories).

Protein samples were loaded onto a focusing tray and the thawed IPG strips were placed gel side down onto each sample. An Isoelectric Focusing apparatus (IEF) (BioRad) was programmed previously according to instruction and the IPG strips were allowed to run overnight. Each strip was removed from the IEF, blotted, and transferred to an equilibration tray. The strips were covered with 2 ml Equilibration Buffer I (BioRad Laboratories) and placed on a rocker for 15 minutes. The equilibration step was repeated with Equilibration Buffer 2 (BioRad Laboratories). The strips were blotted then rinsed in Tris-Glycine-SDS (TGS) running buffer. Each IPG strip was placed into the well of a precast gel cassette (12.5% concentration) using flat pliers until complete contact was made between the strip and the bottom of the gel. A protein size standard was loaded and the gels were run at 10mA until the dye front reached the bottom of the gel. The gel was placed in a fixative solution (10% methanol, 7% acetic acid) and rocked for 1 hour. The fixative was removed and the gel stained in SYPRO Ruby Stain (BioRad Laboratories) overnight. The stain was removed and the gel was destained for 1 hour. The gel was washed 3 times with dH_2O and imaged using a UV transilluminator.

Comparisons between the gels were made and unique spots excised. The excised spots were placed in 50 ml of sterile dH_2O and agitated until dissolved. The tubes were stored at -4°C until sent off for mass spectrometry analysis. The results of this analysis were analyzed using NCBI BLAST to determine similarity to the region E protein.

Results

Preliminary Characterization

According to BLAST searches, the region E gene was found to have homology with a putative hemagglutinin and cell surface proteins found in *B. melitensis*, *B. suis*, *B.*

ovis, *B. canis*, *B. ceti* and *B. pinnipedialis*. Examination of the genes surrounding region E on the genome showed coding regions for putative transposases or putative transposase subunit proteins upstream, downstream, and overlapping the region E gene (Figure 5), indicating that the putative gene is monocistronic and may be a pathogenicity island delivered to *B. melitensis* by horizontal gene transfer.

Cell Lysate Hemagglutination Assay

Sonicated lysates for RB51, RB51-QAE, DH5-α, and DH5-α-TOPOE were used in a series of hemagglutination assays using caprine erythrocytes. Each plasmid, both pQAE and TOPOE, were sequenced and the presence of the region E gene in each was confirmed. Each of the lysates were serially diluted in PBS and equalized in density using spectrophotometric analysis. A 1 ml portion of each lysate was centrifuged and the supernatant was drawn off and used in a hemagglutination assay. Caprine erythrocytes were washed and a 2% erythrocyte solution was prepared on the day of assay preparation. A PBS control was included for both lysate and supernatant assays. Each assay was allowed to incubate at room temperature overnight.

None of the lysates from any species or strain showed any differences in hemagglutination capabilities. Each differed from the PBS control because of the presence of cellular debris in the well. A dark button of erythrocytes was seen in each well, under the cellular debris. It was concluded that all of the lysates tested were hemagglutination negative. Buttons were also observed in the assays studying the supernatants from each of the lysates, confirming that the supernatants were also hemagglutination negative (Table 3).
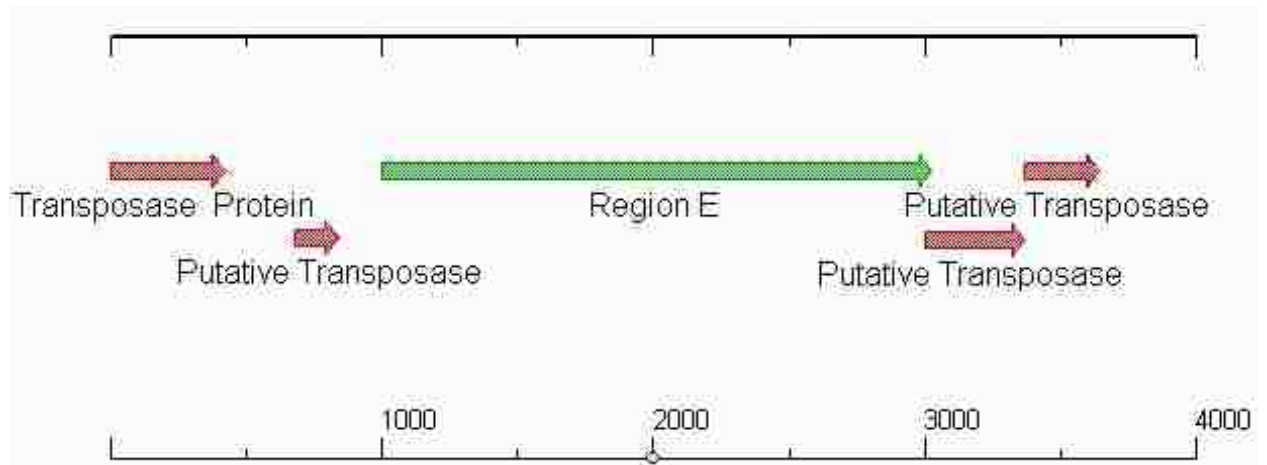
Figure 5.

Map showing the coding regions of the *Brucella melitensis* 16M genome 1000bp upstream and 1000bp downstream of the region E gene.

Table 3. Hemagglutination results observed for RB51, RB51-QAE, DH5-α, and DH5-α-TOPOE using both bovine and caprine erythrocytes.

	Bovine Blood Agar	Caprine Blood Agar	LB Agar	Brucella Broth	Culture Supernatant
Bovine Erythrocytes					
RB51	ND	ND	NP	NP	NP
RB51-QAE	HA+ to 1:8 dilution	HA+ to 1:16 dilution	NP	NP	NP
DH5-α	NP	NP	ND	ND	ND
DH5-α-TOPOE	NP	NP	HA+ to 1:16 dilution	ND	ND
Caprine Erythrocytes					
RB51	ND	ND	NP	ND	ND
RB51-QAE	HA+ to 1:16 dilution	HA+ to 1:32 dilution	NP	ND	ND
DH5-α	NP	NP	ND	ND	ND
DH5-α-TOPOE	NP	NP	HA+ to 1:16 dilution	ND	ND

ND   None Detected
NP   Not Performed

Bacterial Cultures

Broth cultures were prepared for RB51, RB51-QAE, DH5-α, and DH5-α-TOPOE. Both strains of RB51 were cultured in brucella broth. An amount of 45μg/ ml of ampicillin was added to the broth culture of RB51-QAE and to a control culture of RB51. LB broth was used to culture both strains of DH5-α, and DH5-α-TOPOE. An amount of 1μl/ ml of kanamycin was added to the broth culture of DH5-α-TOPOE and to a control culture of DH5-α. A 1 ml portion of each broth culture was centrifuged at high speed and the supernatant was used to prepare serial dilutions in PBS for hemagglutination assays. The broth cultures were equalized using spectrophotometric data and serial dilutions were prepared in sterile PBS. Fresh preparations of 2% caprine and bovine erythrocytes were used.

Clarity and size of button were similar across RB51 and RB51-QAE for both species of erythrocytes tested. The only difference appeared to be button color differences that corresponded to the presence or absence of region E protein. The buttons observed for the RB51-QAE cultures were dark red in color, which differed from those observed for the last 5 dilutions of RB51 and all of the dilutions of PBS, which were a lighter red in color. However, because of the presence of these buttons, all assays for both RB51 strains using both caprine and bovine erythrocytes were considered hemagglutination negative. There appeared to be no visible difference between the control PBS dilutions and the supernatants from both RB51 and RB51-QAE cultures. All supernatant cultures were considered hemagglutination negative.

For cultures of DH5-α and DH5-α-TOPOE, there were no visible differences between strains in either color or clarity across both caprine and bovine erythrocyte

preparations. A button was observed for each dilution of culture as well as for each sample of supernatant tested. All DH5-α and DH5-α-TOPOE cultures and supernatants were considered hemagglutination negative (Table 3).

Bacterial Harvests

Fresh bacterial lawns of RB51, RB51-QAE, DH5-α, and DH5-α-TOPOE were grown on selective media, harvested in sterile PBS, and subjected to hemagglutination assays using both caprine and bovine 2% erythrocyte preparations. RB51 and RB51-QAE were grown on brucella selective blood agar plates. Two series of plates were used to grow both RB51 strains with one series produced using caprine blood and the other produced using blood from a bovine. Ampicillin was added to the plates used to culture RB51-QAE. DH5-α and DH5-α-TOPOE were grown on LB agar plates. Kanamycin was added to the plates used to culture DH5-α-TOPOE. The harvests were equalized in density according to spectrophotometric analysis.

Using caprine erythrocytes, a clear difference was seen between the hemagglutination assays for RB51 and RB51-QAE. Cellular sediment was seen in each well across all dilutions for both strains. A clear demarcation between RB51 cells and erythrocytes was observed as a dark ring of erythrocytes atop a light mass of cells across dilutions. For the sediment of RB51-QAE, there was no demarcation between cells and erythrocytes. Instead, a uniform mass was observed for the first 5 dilutions of RB51-QAE harvests, which were considered hemagglutination positive (Figure 6).

Using bovine erythrocytes, similar results in button consistency were seen for both RB51 throughout each dilution and for RB51-QAE for the first 4 dilutions. For this reason, the first 4 dilutions of RB51-QAE were considered hemagglutination positive.

However, these differences were not as prominent as seen in the caprine erythrocytes. For example, even though there was a more uniform grouping of cells for RB51-QAE, there was still a hint of a light button present, which was not observed in the assays using caprine erythrocytes.

Clear differences were observed between the RB51-QAE harvests for across caprine and bovine erythrocytes. The caprine erythrocyte samples for RB51 appear similar to the bovine erythrocyte samples for RB51. However, RB51-QAE in caprine erythrocytes shows more hemagglutination properties to a greater extent (1 full dilution) than RB51-QAE in bovine erythrocytes. These results were consistent across groups of cells grown on both caprine and bovine blood agar plates (Table 3).

Cell harvests for DH5-α and DH5-α-TOPOE performed similarly in hemagglutination assays as RB51 and RB51-QAE, respectively. The same demarcation effect between erythrocytes and cells was observed for DH5-α dilutions across both caprine and bovine erythrocytes. The first 4 dilutions of DH5-α-TOPOE harvests resulted in a uniform mass of cells with no clear division between both caprine and bovine erythrocytes and bacteria. These first 4 dilutions of DH5-α-TOPOE harvests were considered hemagglutination positive for both bovine and caprine hemagglutination assays. There was no clear difference between the assays using bovine and caprine erythrocytes for DH5-α and DH5-α-TOPOE (Table 3).

Protein Characterization using Absorption Assay and 2D Gel Electrophoresis

For the absorption analysis assay, serum from goats inoculated with Brucella species expressing the region E gene (2308-QAE, RB51-QAE and 16M) was absorbed with the lysates from corresponding Brucella species lacking the region E gene (2308,
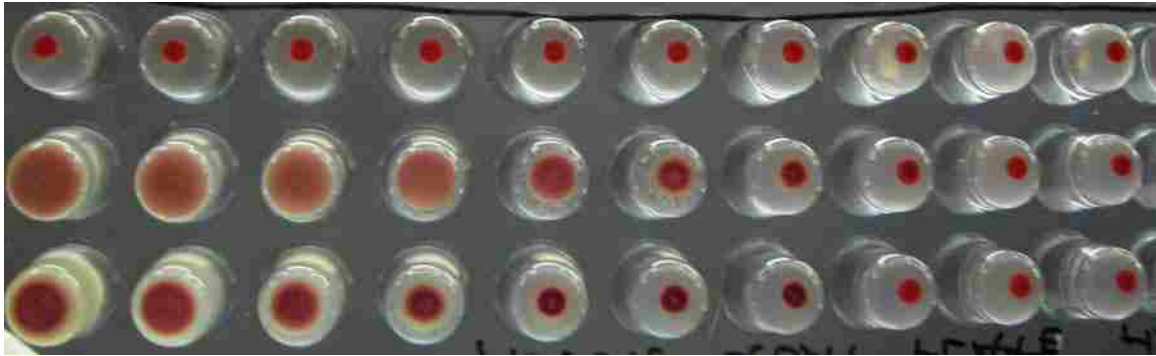
Figure 6.

Assay comparing the hemagglutinating capabilities of RB51 and RB51-QAE using caprine erythrocytes.   Each sample was subjected to 1:2 serial dilutions made in sterile PBS and mixed with 100μl washed caprine erythrocytes.

Row 1: Sterile PBS control
Row 2: RB51-QAE cell harvests
Row 3: RB51 cell harvests

RB51, and 16MΔE). A unique band was observed on western immunoblots run with the serum of goats inoculated with species of *Brucella* containing the region E gene that was not present in those lanes containing the lysates of *Brucella* species lacking the region E sequence. This band was approximately 74 kD in size, which is the approximately the estimated size of the region E putative hemagglutinin protein. This unique banding was seen especially for the RB51-QAE lysates on the immunoblot (Figure 7). Considering that RB51-QAE is a rough strain, this effect may be due to increased exposure of the region E protein due to a lack of OPS interference.

Lysates of RB51 and RB51-QAE as well as cell membrane isolates of DH5α and DH5α-TOPOE, were analyzed using 2D electrophoresis. The resultant RB51 and RB51-QAE gels were compared and a unique spot could not be identified (Figure 8). Because of the complexity of the whole cell lysates, outer membrane fractions of DH5α and DH5α-TOPOE were collected and subjected to 2D analysis (Figure 9). A single spot with an isoelectric point and molecular weight of the region E hemagglutinin was identified in DH5α-TOPOE, but mass spectrometric analysis of the sopt failed to confirm a peptide map that matched.

Discussion

Genomic comparisons have been made between the region E protein and a 31kD outer membrane protein found on the surface of *B. suis* (omp31), which has been described as a cell surface heme-binding protein. Hemagglutination assays were prepared to test the hemagglutination ability of RB51 versus RB51-QAE. It was expected that RB51-QAE would have an increased effect on hemagglutination, as the region E gene encodes for a putative hemagglutinin. Assays showed that at high
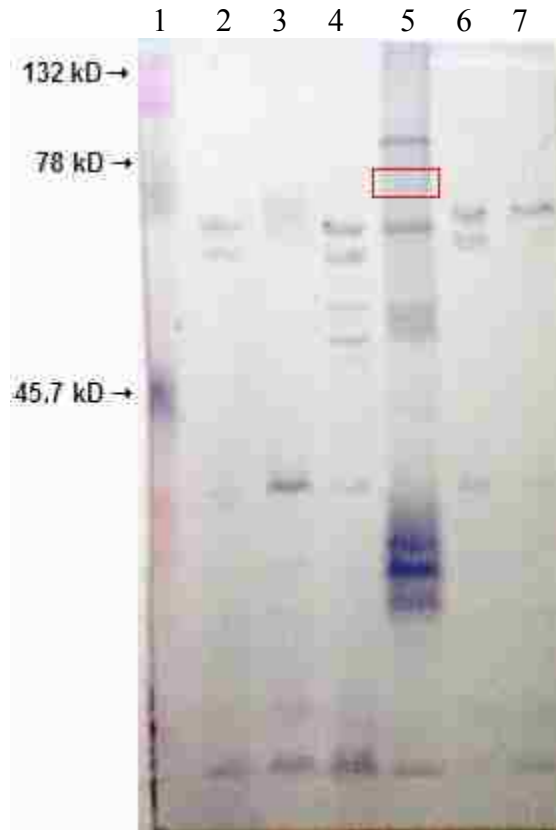
Figure 7.

Western Blot run with absorbed serum from a goat inoculated with RB51-QAE. The serum was absorbed using RB51 previously. The unique banding pattern seen in the lane containing RB51-QAE lysates indicates that the region E protein is capable of eliciting a specific humoral response in goats when expressed by RB51. The unique bands are seen between approximately 60 and 80 kD, according to the size standard in lane 1. Because the estimated size of the region E protein is 68.94 kD, it is proposed that one of these unique bands (designated by the red box) indicates antibodies specific for the region E protein. The bands are observed in lanes 3 and 7 as well, but were too faint to appear using our best imaging techniques. This may be due to phenotypic differences between 16M and 2308-QAE as compared to RB51-QAE.

The lane order is as follows:

1. Size standard marker
2. *B. abortus* 2308
3. *B. abortus* 2308-QAE
4. *B. abortus* RB51
5. *B. abortus* RB51-QAE
6. *B. melitensis* 16MΔE
7. *B. melitensis* 16M

2D image of *B. abortus* RB51
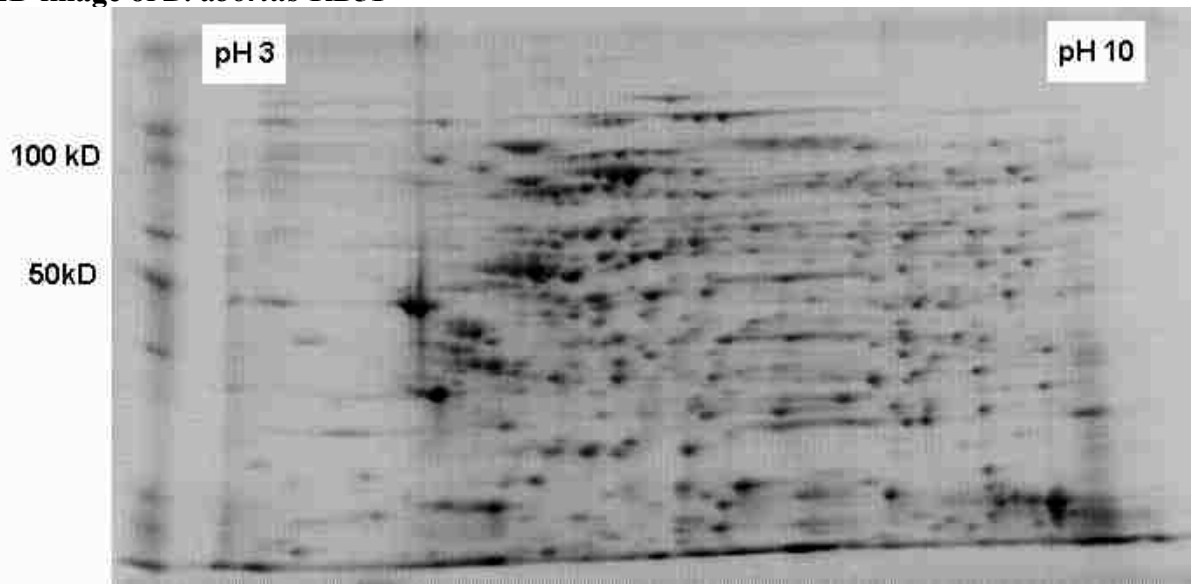
2D image of *B. abortus* RB51-QAE
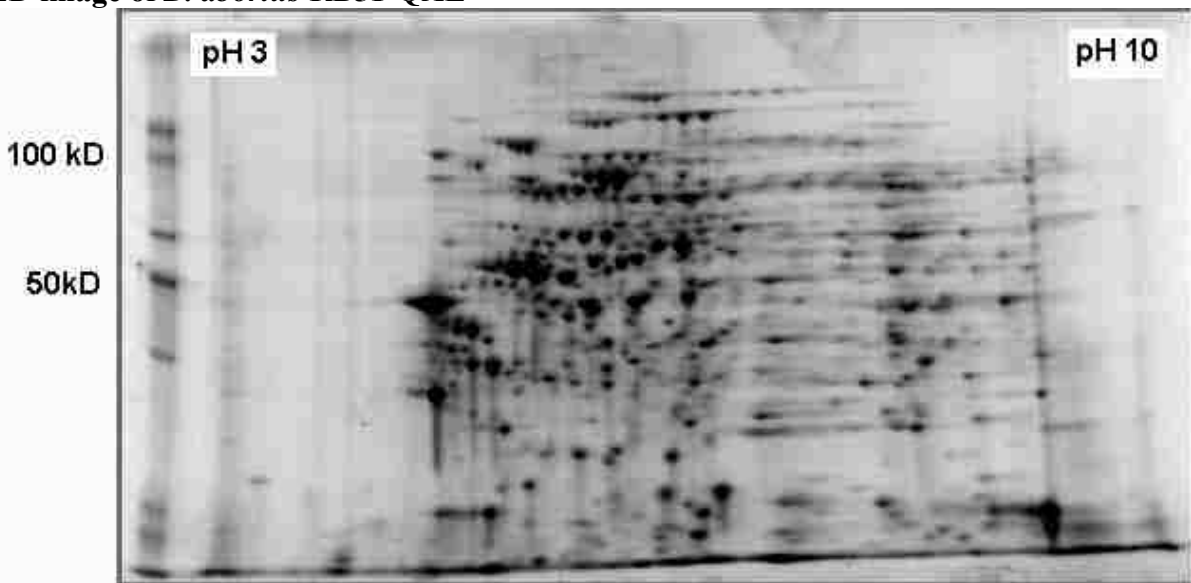
Figure 8.

2D gel images taken of *B. abortus* RB51 and *B. abortus* RB51-QAE for the purpose of
identifying unique proteins produced by RB51-QAE. The complexity of the 2D gel
images of *B. abortus* RB51 and *B. abortus* RB51-QAE precluded the identification of a
unique spot for RB51-QAE that corresponded to the region E hemagglutinin. The top
image is of the 2D gel run with RB51 lysates and the bottom image is of RB51-QAE.

2D image of DH5α isolated membrane
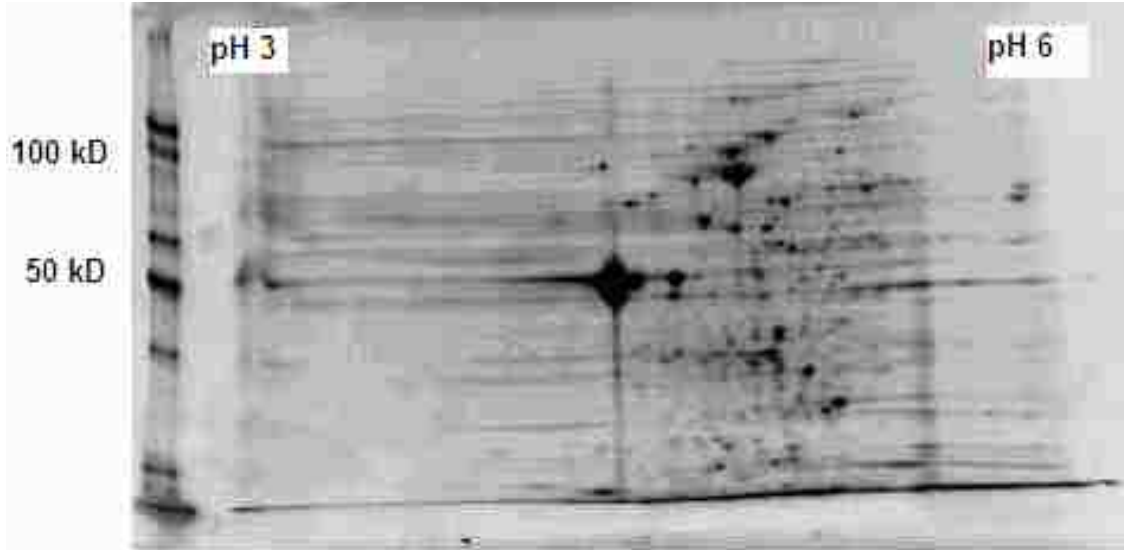
2D image of DH5α-TOPOE isolated membrane

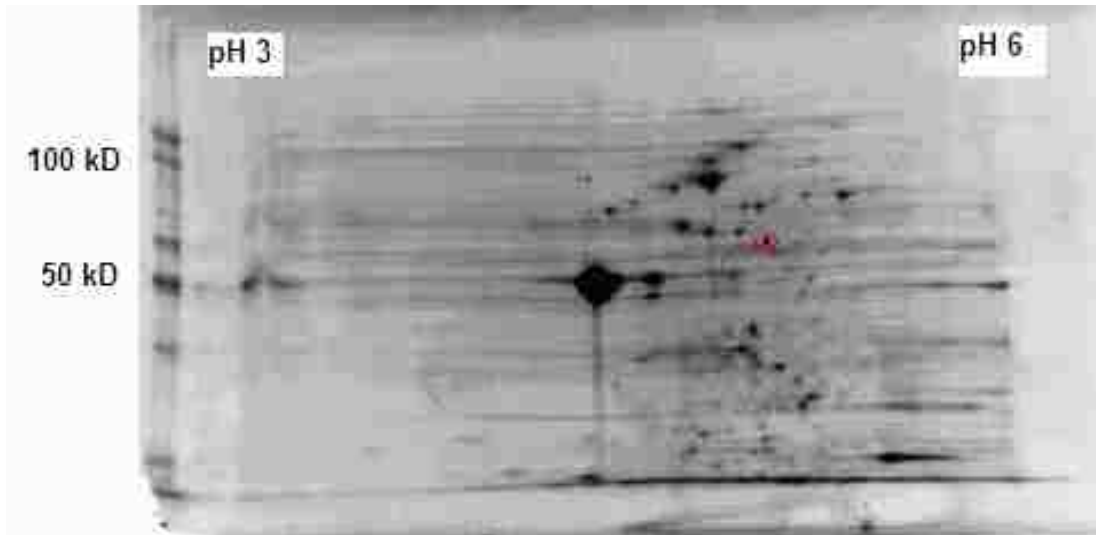**Figure 9.**

2D gel images were taken of *E. coli* DH5α and DH5α-TOPOE for the purpose of identifying unique proteins produced by DH5α-TOPOE. The top image is of the 2D gel run with DH5α membrane isolates and the bottom image is of DH5α-TOPOE membrane isolates. The unique protein identified on the DH5α-TOPOE gel is outlined in red and was analyzed by mass spectrometry.

numbers, presence of the region E gene in the genome of RB51-QAE caused positive hemagglutination in a 2% bovine and caprine erythrocyte solutions, whereas RB51 did not. Furthermore, a higher incidence of hemagglutination was observed when subjected to the assay using erythrocytes from goat blood, as compared to blood erythrocytes from a cow. Also, a greater incidence of hemagglutination was seen when the RB51-QAE tested was grown on media using goat blood rather than cow blood. This suggests that not only is the region E protein a hemagglutinin, but it is also a host-specificity factor. The hemagglutination assays for *E. coli* DH5α and DH5α-QAE were similar in that positive hemagglutination was noted for the region E mutant, and not for the parental strain. No agglutination was observed using cell broth culture supernatant for any of the bacterial strains tested, suggesting that the hemagglutinating factor was not excreted from the cell, but present on the surface. Further studies would be needed on a proteomic level to further characterize the cellular location, hemagglutination and host specificity capabilities of the region E protein.

The absorption assay performed indicated unique bands on western immunoblots run with serum from goats inoculated with various strains of brucella both containing and lacking the region E gene sequence. These unique bands, at approximately 74kD, are of the approximate size of the putative hemagglutinin encoded by the region E sequence. These bands were not present in the blot lanes for those strains that did not contain region E. Specifically, this suggests that the immunogenic protein produced by 2308-QAE, RB51-QAE, and 16M is recognized by the goat immune system and is not produced by 2308, RB51, and 16MΔE. This indicates that the putative hemagglutinin may increase the immunogenic capabilities of RB51 in the host animal,

inducing the production of anti-region E protein antibodies. This is another finding that supports the proposal that RB51-QAE would be an improved vaccine candidate when compared to RB51.

There was a measureable difference in hemagglutination in isolates carrying the region E clone, but the protein could not be identified in the 2D analysis. This could be due to many factors, such as the region E protein being the same size and isoelectric point as another RB51 protein or cluster or proteins, which would make the region E protein indistinguishable from the other proteins. The 2D analysis of the pure protein would be beneficial, but the exact size and isoelectric point of the region E protein are estimated at this time, and not certain.

CHAPTER THREE

EVALUATION OF PCR AS A DIAGNOSTIC TOOL FOR BRUCELLOSIS

Introduction

The control of brucellosis as a worldwide disease relies upon effective

vaccination in animals and proper diagnostic testing. Human brucellosis, most often

contracted through interaction with infected animals, remains problematic in many

countries around the world. Because there is no vaccine for human use, control of human

brucellosis depends on accurate diagnosis and proper treatment. Currently, diagnosis of

brucellosis in humans involves serologic testing and confirmation through blood culture.

Although this is the diagnostic gold standard, there are issues involved with these

techniques that may hinder proper diagnosis. Serologic tests are subject to false-positive

results due to cross reacting antibodies and confirmation through culture may take weeks,

as *Brucella spp*. are slow growing organisms.

Methods of detection utilizing the polymerase chain reaction (PCR) are being

investigated for improved diagnosis. A reliable PCR technique would be quicker, easier,

and more affordable than current procedures while eliminating the possibility of false

positive reactions. Current research in this area, however, has been inconsistent. Results

differ based on biological sample type tested, DNA isolation methods used, and variation

in testing schedules.

In this study, the limit of detection of *B. abortus* 2308 and *B. melitensis* 16M was

examined using the Baily, Omp25, and ORF-944 *Brucella*-specific primer sets for

conventional PCR amplification and visualization using agarose electrophoresis. These

laboratory strains were diluted in fluid samples that may be taken for diagnostic purposes,

including blood, plasma, serum, and urine. Optimization of DNA isolation technique and thermalcycler protocols was also examined. The results of this study can aid in continuing efforts to develop a reliable PCR technique for the purpose of improved diagnosis of human brucellosis and increased control of the disease worldwide.

Materials and Methods

Bacterial Strains

    B. abortus 2308 and *B. melitensis* 16M were studied in these PCR assays. Bacteria were obtained using stocks of 1×10^{10} (2308) and 1×10^{11} (16M) colony forming unit (CFU) concentrations and chloroform-killed. These cells were then serially diluted to 1×10^{1} CFU in sterile H_2O, whole blood, plasma, serum, or urine and subjected to either direct PCR amplification or DNA isolation and quantification.

    One assay for the limit of detection of the region E gene sequence using the region E primer set was performed using B. abortus RB51-E and 16M as template. For this assay, bacteria were obtained as described above from previously prepared stocks of 1×10^{9} CFU for RB51-QAE and 1×10^{11} CFU for 16M. These cells were serially diluted ten-fold to 1×10^{1} CFU in sterile H_2O and subjected to PCR amplification using the region E ORF-944F and ORF-944R primers following DNA isolation and quantification.

DNA Isolation

    Two separate DNA isolation protocols were performed on the cellular dilutions made in whole blood. The first protocol used was outlined in the Qaigen DNeasy Blood and Tissue Handbook (Qaigen, Inc.) using the Qaigen DNeasy Blood and Tissue Kit (Qaigen, Inc.). The second protocol used is the standard protocol used in lab, which also utilizes the Qaigen DNeasy Blood and Tissue Kit (Qaigen, Inc).

The first DNA isolation protocol was performed by mixing 50μl of a blood dilution with 180μl buffer ATL (Qiagen, Inc.). A volume of 20μl of proteinase K was added to the mixture, vortexed, and placed in a shaking water bath at 55°C for 1 hour. Two hundred μl of buffer AL was added and the tubes were placed in a 70°C heating block for 10 minutes. Two hundred microliters of 100% ethanol was added and the samples were placed into a DNeasy spin column. A volume of 500μl of buffer AW1 was added to the sample and centrifuged at 8000rpm for 1 minute. Five hundred μl of buffer AW2 was added and the samples were spun at 14,000rpm for 3 minutes. The spin column was placed into a clean 1.5 ml centrifuge tube, 200 μl of buffer AE was pipetted onto the membrane of the spin column and allowed to incubate at room temperature for 1 minute. The samples were centrifuged at 8000 rpm for one minute. A second elution step was repeated. The resultant isolated DNA was stored at 20°C until use.

The second DNA isolation protocol was performed by pipetting 50μl of a blood dilution sample into a 1.5 ml centrifuge tube containing 20μl of proteinase K (Qiagen, Inc.). The volume was adjusted to 220μl with sterile PBS (Sigma Chemical Co.). A 200μl volume of buffer AL (Qiagen, Inc.) was added to the tube, mixed by vortexing, then incubated with agitation at 56°C for 10 minutes. Next, a volume of 200μl of 100% ethanol was added, the mixture was loaded into a DNeasy spin column (Qiagen, Inc.), and centrifuged at 8000 rpm for 1 minute. Five hundred μl of buffer AW1 (Qiagen, Inc.) was added and the column was centrifuged at 8000rpm for an additional minute. Next, 500μl of buffer AW2 (Qiagen, Inc.) was added to the spin column and the samples were centrifuged at 14,000rpm for a period of 3 minutes. The spin column was placed into a clean 1.5 ml microcentrifuge tube and 200μl of buffer AE (Qiagen, Inc.) was added to the

tube and allowed to incubate at room temperature for 1 minute. A second elution step was repeated for maximum DNA collection. The tube was centrifuged for 1 minute at 8000rpm and the resultant flow-through was stored at 20°C until use.

The remaining groups (dilutions prepared in sterile H2O, plasma, urine, and serum) were subjected to DNA isolation using only the first DNA isolation protocol, as it proved to be a better technique for maximum isolate DNA collection.

Primer Selection and Preparation

The Omp25 primer set was designed using the Omp25 gene sequence of *B. abortus,* which is highly conserved across *Brucella* spp., by Phillipe de Wergifosse et al. (de Wergifosse et al., 1995). The first set of primers selected targets the Omp25 gene and are designated Omp25F (5' –GTTCCGGCTCCGGTTGAAGTA -3') and Omp25R (5' – CGCGGATATCCTGCGTGTC -3'). The second set of primers were specific for 31 kDa *B. abortus* antigen and designed to detect both *B. abortus* and *B. melitensis* (Baily et al., 1992). The Baily primers were designated B4 (5' – TGGCTCGGTTGCCAATATCAA- 3') and B5 (5'- CGCGCTTGCCTTTCAGGTCTG- 3'). While both primer sets are used to detect *B. abortus* and *B. melitensis* DNA, neither will differentiate between the two species. A third set of primers was used for a limit of detection assay targeting the region E gene sequence in 16M and RB51-QAE. The ORF-944F and ORF-944R primers were used as previously described. Each primer set was synthesized by Integrated DNA technologies, Inc. (Coralville, IA) and re-suspended using PCR grade H₂O upon arrival.

Limit of Detection

Preliminary studies were performed using previously isolated DNA from *B. abortus* 2308, *B. abortus* RB51-QAE and *B. melitensis* 16M. DNA was quantified using

the GE Healthcare RNA/DNA Calculator Spectrophotometer Model Gene Quant Pro (Amersham Bioscience). A series of 1:10 dilutions were prepared in sterile H_2O and subjected to PCR using the Omp25 and Baily primer sets. A second set of 1:10 dilutions was made for both *Brucella* species, but using known concentrations. Specifically, a starting concentration of 1×10^{11} CFU/ml of *B. melitensis* 16M and a 1×10^{10} CFU/ml starting concentration of *B. abortus* 2308 were both diluted ten-fold to a concentration of 1×10^1 CFU/ml in sterile H_2O. These preparations were also subjected to PCR using both primer sets. Using the same 16M dilutions (1×10^{11} CFU/ml to 1×10^1 CFU/ml in sterile H_2O) and a set of similarly prepared RB51-QAE dilutions (1×10^{11} CFU/ml to 1×10^1 CFU/ml in sterile H_2O), a limit of detection assay was performed using conventional PCR and the region E ORF-944 primers. The resultant amplifications were subjected to agarose gel electrophoresis and visualized as previously described.

Bacterial Sample Preparation

A total of 5 experimental groups were prepared for PCR analysis based on the fluid used to dilute each of the samples. The first group studied the PCR detection capabilities of the Omp25 and Baily primers on 2308 and 16M using colony forming units (CFU) as template and isolated DNA diluted in sterile H_2O. The detection capabilities of the ORF-944 primers on RB51-QAE and 16M using DNA isolated from CFU dilutions in sterile H_2O was also studied. For all groups, samples were prepared following chloroform killing. Serial dilutions of 1:10 were prepared in sterile H_2O for each *Brucella* species and each dilution was subjected to PCR individually. A 1.0 μl portion was used as PCR template and the rest subjected to DNA isolation. The results from this first group were used as a control for comparison of the remaining four groups.

The next group consisted of chloroform killed CFUs from both 16M and 2308 diluted in whole blood. A series of 1:10 dilutions were made as described above with both 2308 and 16M, but using whole blood obtained from an infection-free animal in the place of sterile H_2O. Similarly, a 1.0 μl sample of each dilution was used directly for PCR and the rest underwent DNA isolation.

The remaining groups were prepared in a similar fashion, except using plasma, urine, or serum in the place of whole blood. Each group was subjected to PCR using CFU as template and to DNA isolation to use for PCR as a DNA template.

PCR Technique

PCR was performed as described previously using the FailSafe PCR System with 2X G Premix (Epicentre Biotechnologies) and a MyCycler Personal Thermal Cycler (BioRad Laboratories). For amplification using the Omp25 primers, template DNA was subjected to an initial denaturation step for 4 minutes at 95°C. Next, 34 cycles were completed with denaturation at 95°C for 30 seconds, primer annealing at 65°C for 30 seconds, and elongation at 72°C for 2 minutes. A final elongation stage of 72°C was performed for 10 minutes (Edmonds 2001). For amplification of the Baily primers, template DNA was subjected to an initial denaturation step for 5 minutes at 95°C. Then 40 cycles were completed with denaturation at 90°C for 1 minute, primer annealing at 60°C for 30 seconds, and elongation at 72°C for 1 minute. A final elongation stage of 72°C was performed for 7 minutes. A second series of PCR assays were performed using the same PCR preparations, but elongating each of the primer thermal cycler protocols by 5 cycles in an attempt to optimize amplification. This brought the total number of cycles to 39 for the Omp25 protocol and 45 for the Baily protocol for these asssays (Baily

1992). For amplification using the ORF-944 primers, the thermal cycler protocol was performed as previously described. The resultant PCR amplifications were subjected to Agarose gel electrophoresis for visualization as previously described.

Results

DNA quantification

Using previously made *B. abortus* 2308 and *B. melitensis* 16M stocks, a series of nine 1:10 dilutions (ranging from approximately 1 μg/μl to 1×10^{-8} μg/μl) were prepared for each in 100μl sterile H_2O. The standard laboratory DNA isolation protocol described previously was used to isolate DNA from each dilution. The DNA isolates of only 100% concentration were quantified, as any dilutions were not quantifiable by the equipment used. For the pure 2308 sample, a DNA quantity of 0.87 μg/μl was measured. For the pure 16M sample, a quantity of 0.94 μg/μl was measured. The RB51-QAE sample yielded a DNA quantity of 0.4x102 μg/μl.

The PCR amplification performed using the Omp25 primers and 16M DNA provided a limit of detection of $1\times10^{-4.05}$, or 1:1000, of the original sample, which is approximately 9.35x10^{-5} μg/μl. The PCR amplification performed using the Omp25 primers and 2308 DNA provided a limit of detection of approximately 8.72x10^{-6} μg/μl, which is a 10^{-5} dilution of the original sample.

The PCR amplification performed using the Baily primers and 16M DNA provided a limit of detection of $1\times10^{-7.4}$ of the original dilution, which is approximately 9.35x10^{-8} μg/μl. The PCR amplification performed using the Baily primers and 2308 DNA provided a limit of detection of 1×10^{-7} of the original dilution, which is approximately 8.72x10^{-8} μg/μl (data not shown).

Using the ORF-944 primers and 16M DNA, a limit of detection was found to be approximately 9.35×10^{-5} µg/µl. For the ORF-944 primers ad 2308 DNA, a limit of detection was found at 4.0×10^{-6} µg/µl (Figure 10).

Thermal Cycler Protocol Optimization

In an attempt to optimize the standard thermal cycler protocols for both the Baily and Omp25 primers, preliminary PCR assays were performed using the standard number of amplification cycles for each primer set and an elongated protocol using 5 additional amplification cycles for each primer set. It was observed that the elongated protocol provided greater detection capabilities for both 16M and 2308 CFU and DNA than the standard thermal cycler protocol for both primer sets. Specifically, for the PCR assays using both 2308 and 16M CFU as template, the original thermal cycler protocol provided a limit of detection at 1×10^8 CFU/ ml and the elongated thermal cycler protocol provided a limit of detection at 1×10^5 CFU/ ml (for both 16M and 2308 using both primer sets). Because of these results showing a 1000-fold increase in detection, the elongated protocols were used for these PCR experiments and would be recommended for further experimentation on the detection of *Brucella* cells using conventional PCR.

B. abortus 2308 and B. melitensis 16M in Sterile H$_2$O

The first series of PCR were performed using DNA isolated from a known amount of colony forming units (CFU) of both 2308 and 16M as template, as well as using CFU as template using the Omp25 and Baily primers for both. The CFU were diluted in sterile H$_2$O for a series of ten 1:10 dilutions from the original 1×10^{10} concentration of 2308 CFU and eleven 1:10 dilutions from the original 1×10^{11}
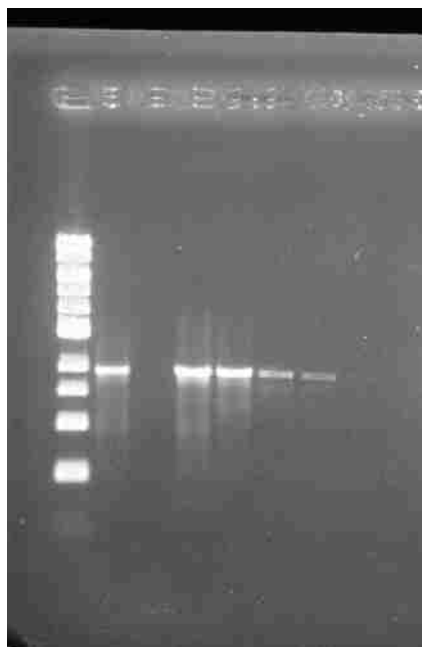
Figure 10.

Agarose gel electrophoresis of the PCR amplification products of 16M DNA serially diluted in sterile H_2O using the ORF-944 region E primers.

The lane order for the 16M gels is as follows:

1. 1kb size standard marker
2. Positive RB51-QAE control
3. Negative control
4-9. RB51-QAE DNA concentrations from 10^7 CFU/µl – 10^2 CFU/µl

concentration of 16M CFU (both diluted to a final concentration of 1×10^1 CFU). A 1.0

µl sample of each CFU dilution was subjected to PCR using both primer sets. The rest of

the dilutions underwent the standard lab DNA isolation procedure described and the

resultant DNA isolates were subsequently amplified by PCR using both primer sets.

Using the Omp25 primers and 2308 CFU, positive identification was possible to

the 10^3 2308 CFU/µl H_2O. Using the Baily primers and 2308 CFU, positive

identification was also possible to the 10^3 2308 CFU/µl H_2O. Faint banding seen on the

gel in dilutions greater than 10^3 2308 CFU/µl H_2O was rare and this was most likely due

to a contamination error. Using both the Omp25 and Baily primers and 16M CFU,

positive identification was possible to the 1×10^3 16M CFU/µl H_2O. There were no faint

bands witnessed in dilutions greater than 1×10^3 16M CFU/µl H_2O like those seen for

2308 (Figure 11).

Using the Omp25 primers and 2308 DNA isolates, positive identification was

possible for every dilution tested (1×10^9-1×10^{01} 2308 DNA/100µl H_2O). The same was

observed using the Baily primers and 2308 DNA isolates. For the DNA isolated from

16M, both the Omp25 and Baily primers produced positive banding for all dilutions

(1×10^{10}-1×10^{01} 16M DNA/100µl H_2O) (Figure 12). Contamination was ruled out for all

PCR reactions due to the absence of a band in the negative control lane.

DNA Isolation Techniques for Whole Blood

Two DNA isolation techniques were tested for optimal DNA recovery from

samples of whole blood spiked with *B. abortus* 2308 and *B. melitensis* 16M. The first

was a standard procedure used in lab for DNA isolation from *Brucella* spp. CFU. The

97
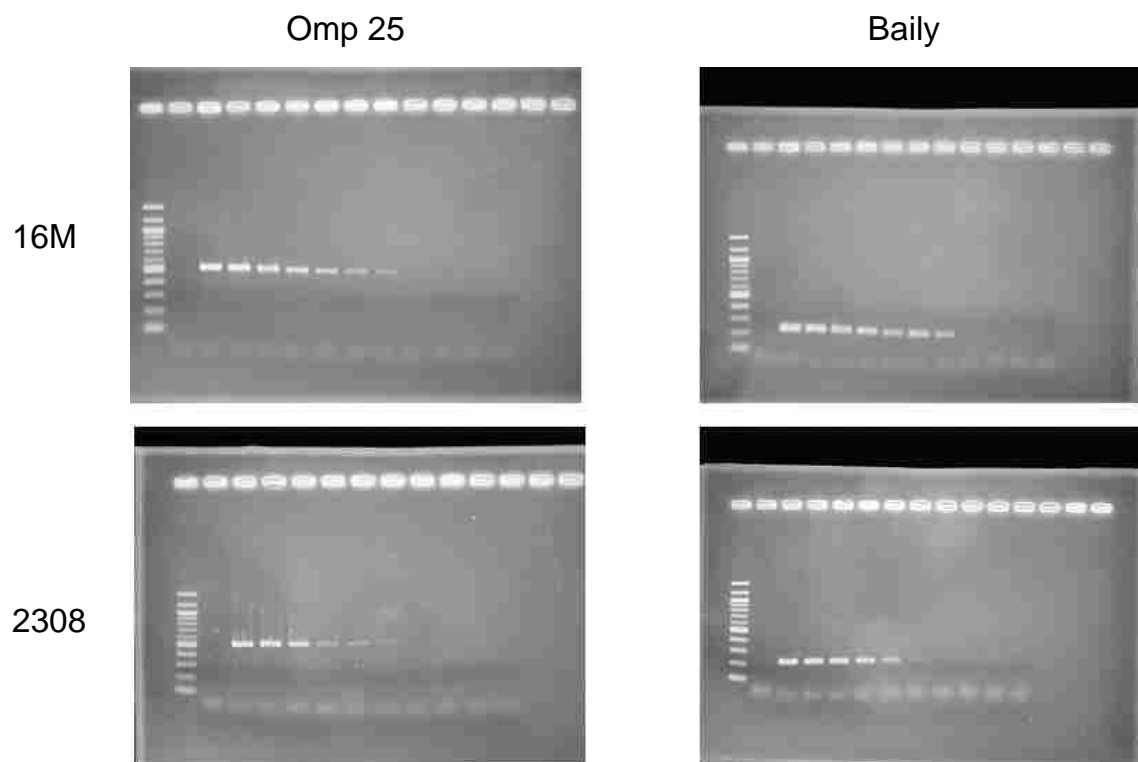
Figure 11.

Results of the agarose gel electrophoresis of the PCR amplification products of 2308 and 16M in sterile H_2O using CFU as template and both primer sets.

The lane order for the 16M gels is as follows:

      1. 1kb size standard marker
      2. Negative control
      3-13. 16M CFU concentrations from 10^9 CFU/μl – 0.1 CFU/μl

The lane order for the 2308 gels is as follows:

      1. 1kb size standard marker
      2. Negative control
      3-13. 2308 CFU concentrations from 10^8 CFU/μl – 0.1 CFU/μl

Omp25   Baily

16M
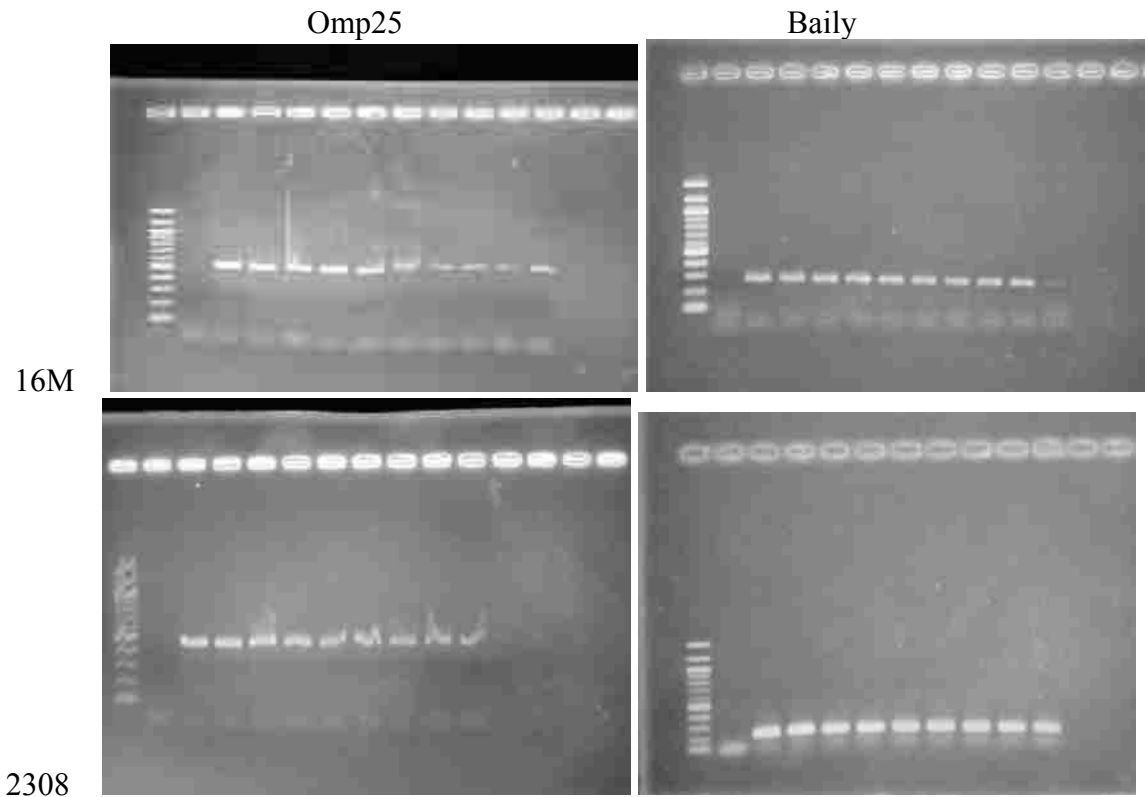
2308

Figure 12.

Agarose gel electrophoresis of the PCR products of DNA isolated from ddH_2O dilutions of 2308 using the Omp25 primer set. These results were consistent with those observed for the amplification of 2308 DNA using the Baily primers and for the amplification of 16M DNA across both primer sets as well.

The lane order for the 16M omp25 and Baily gels is as follows:
   1. 1kb Marker
   2. Negative control
   3-12. DNA isolation concentrations from 10^8 CFU/µl – 1 CFU/10µl

The lane order for the 2308 omp 25 and Baily gels is as follows
   1. 1kb Marker
   2. Negative control
   3-11. DNA isolation concentrations from 10^7 CFU/µl – 1 CFU/10µl

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second was the suggested procedure for DNA isolations from samples of whole blood. Both procedures were taken from the Qiagen DNeasy kit manual.

The first DNA isolation procedure provided a greater output of isolated DNA, as shown by higher detection capabilities when subjected to PCR amplification. As a result, the remaining DNA isolations performed utilized the standard lab procedure first described.

B. abortus 2308 and B. melitensis 16M in Whole Blood

Serial 1:10 dilutions were prepared similarly to the first series of PCR assays, but using whole blood from a brucellosis negative goat in the place of sterile H_2O. The CFU were diluted in whole blood for a series of ten 1:10 dilutions from the original 1×10^{10} concentration of 2308 CFU and eleven 1:10 dilutions from the original 1×10^{11} concentration of 16M CFU. The dilutions underwent both the first and second DNA isolation procedures described and the DNA isolates were amplified by PCR using both primer sets. The DNA examined in these experiments was obtained through DNA isolation of whole blood and CFU mixtures and at no time was DNA alone added to any dilution of whole blood.

Using whole blood to dilute *B. abortus* 2308 and *B. melitensis* 16M CFU as template for PCR using both primer sets proved unsuccessful for detection in any of the dilutions tested. Elongation of thermal cycler protocols for both Omp25 and Baily primers did not improve detection and no positive banding was observed on any of the gels run for these assays (data not shown).

The standard lab procedure for DNA isolation provided greater detection capabilities than using CFU alone when testing whole blood dilutions spiked with 2308 and 16M. Using the *B. melitensis* 16M DNA isolated from CFU in whole blood, the Baily primers provided a limit of detection down to the 1×10^3 original CFU dilution. The Omp25 primers were not as effective for *B. melitensis* 16M DNA and provided positive banding to the 1×10^4 original CFU dilution when diluted in whole blood. For the *B. abortus* 2308 DNA isolates, both primer sets provided positive banding to the 1×10^2 original CFU dilution, but this band was extremely faint. For this reason, it was concluded that the limit of detection for *B. abortus* 2308 DNA isolated from CFU in whole blood was seen at 1×10^3 original CFU dilution (Figure 13).

The second procedure for DNA isolation was not as effective as the standard procedure described above. For the 16M DNA isolates, both Omp25 and Baily primers were able to provide a limit of detection of 1×10^6 original CFU concentration. For the 2308 DNA isolates, both Omp25 and Baily primers provided positive banding up to the 1×10^6 original CFU concentration. However, the band seen for a 1×10^6 CFU concentration of *B. abortus* 2308 DNA was extremely faint for both primer sets, and was not considered sufficiently visible for an absolute positive result. For this reason, it was concluded that the limit of detection for *B. abortus* 2308 DNA using the standard lab protocol for DNA isolation is 1×10^7 original CFU concentration in whole blood for both Omp25 and Baily primer sets.

Both DNA isolation procedures performed for the 2308 and 16M dilutions in whole blood exhibited inferior detection capabilities when compared to those observed for 2308 and 16M dilutions in sterile H_2O. It was noted during DNA isolation for both

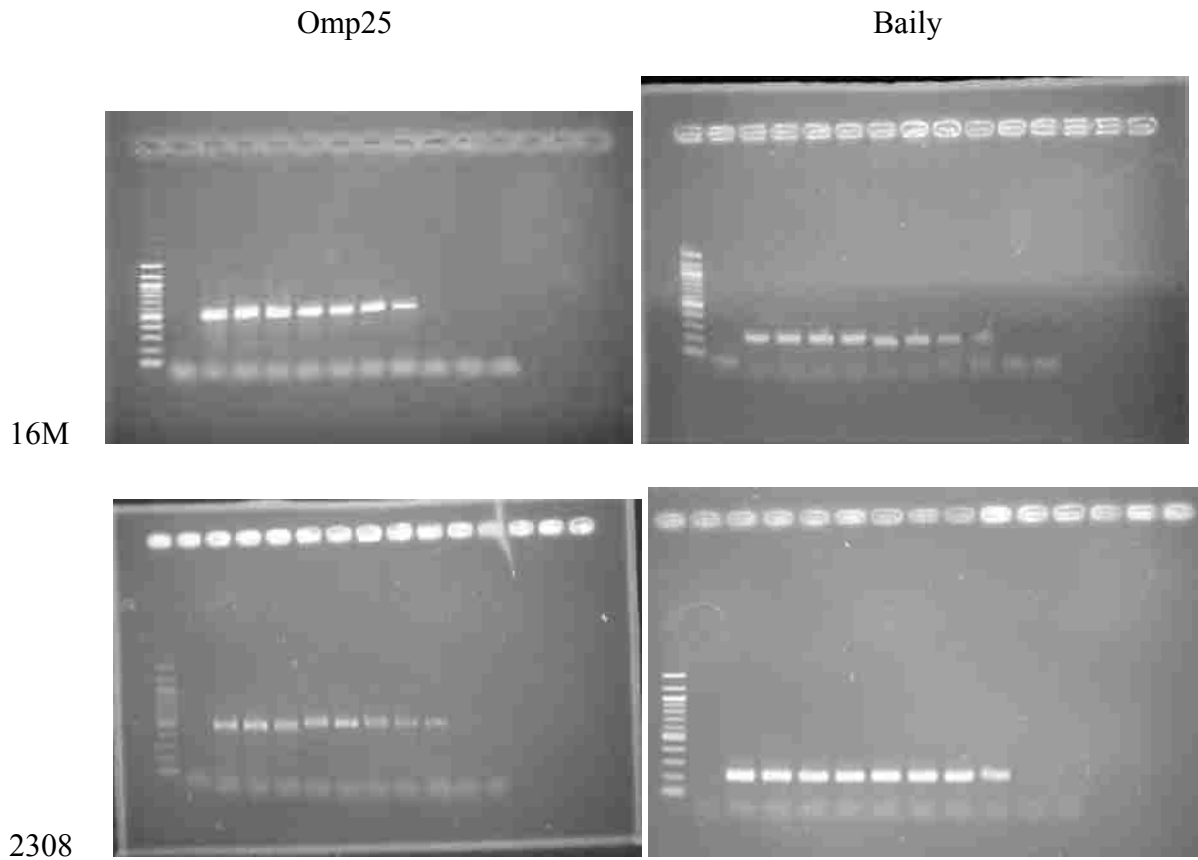Omp25   Baily

16M

2308

Figure 13.

Agarose gel electrophoresis of the PCR products of DNA isolated from dilutions of 2308 and 16M CFU in whole blood using the Baily and Omp25 primer sets.

The order of the lanes is as follows:

1. 1kb size standard marker
2. Negative control
3. DNA isolation concentrations from 10^8 CFU/μl – 1 CFU/10μl

procedures that red blood cell lysis products tend to block flow through a spin column, which may have inhibited the amount of DNA isolated from these samples. For this reason, whole blood would not be considered as an ideal fluid for the detection of *Brucella* cells, if present in the sample (Not pictured).

B. abortus 2308 and B. melitensis 16M in Plasma

Serial 1:10 dilutions were prepared in the same way as the whole blood series of PCR assays, but using plasma from a brucellosis negative goat in the place of blood. The *B. melitensis* 16M and *B. abortus* 2308 CFU were diluted similarly. The dilutions underwent only the first standard DNA isolation procedure described and the DNA isolates were amplified by PCR using both primer sets.

For the PCR amplification of *B. melitensis* 16M CFU, a limit of detection was observed at a concentration of 1×10^5 CFU using both the Omp25 and Baily primers. Using *B. abortus* 2308 CFU as template for both primer sets yielded a limit of detection of 1×10^5 CFU (Figure 14).

Amplification of DNA isolated from plasma dilutions yielded positive detection in both species of *Brucella*, in all dilutions, using both primer sets. No contamination was detected, as the negative control lane did not produce banding. Based on these results, it would be possible to detect DNA isolated from CFU of 16M or 2308, if present, in a sample of plasma using the Omp25 and Baily primers for conventional PCR (Figure 15).

B. abortus 2308 and B. melitensis 16M in Serum

The PCR assays for serum spiked with 2308 and 16M CFU provided results similar to those for 2308 and 16M CFU dilutions in sterile H_2O for both primer sets. Detection of CFU diluted in serum yielded a limit of 1×10^3 CFU/µl in both 16M and

Omp 25   Baily

16M
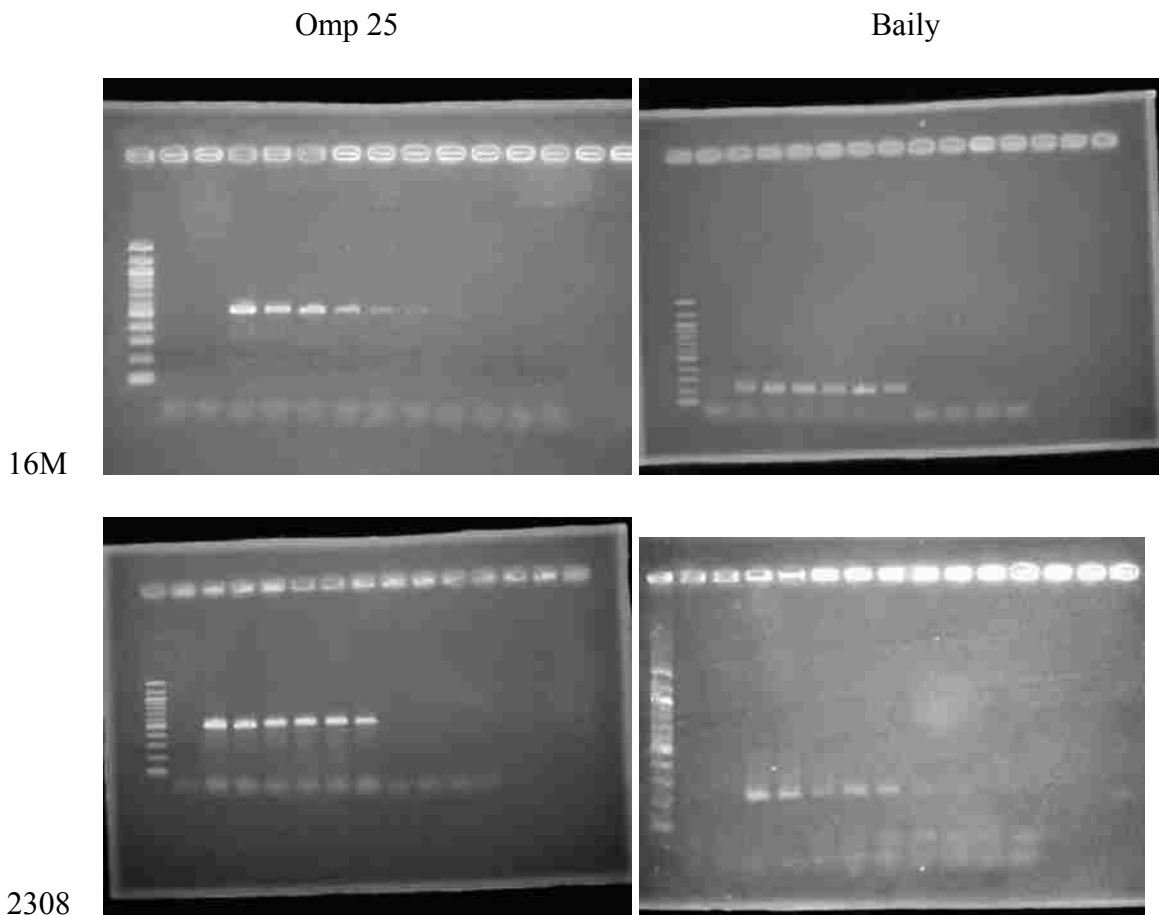
2308

Figure 14.

Results of the agarose gel electrophoresis of the PCR amplification products of 2308 and
16M in plasma using CFU as template and both primer sets.

The lane order for the gels is as follows:

1. 1kb size standard marker
2. Negative control
3-12. CFU concentrations from 10^8 CFU/μl – 0.1 CFU/μl

Omp25   Baily

16M

2308

Figure 15.

Agarose gel electrophoresis of the PCR products of DNA isolated from dilutions of 16M and 2308 CFU in plasma using the Baily and Omp25 primer sets. Detection is possible in every dilution.

The order of the lanes for the Omp25 gels is as follows:

      1. 1kb standard size marker
      2. Negative control
      3-11. DNA isolation concentrations from 10^7 CFU/μl – 1 CFU/10μl

The order of the lanes for the Baily gels is as follows:

      1. 1kb standard size marker
      2. Negative control
      3-12. DNA isolation concentrations from 10^8 CFU/μl – 1 CFU/10μl

2308 using both Omp25 and Baily primers (Figure 16). DNA isolated from serum

dilutions yielded positive detection following PCR amplification in all 16M and 2308

dilutions ($1x10^9$-$1x10^1$ original CFU concentration) using both primer sets (Figure 17).

These results show that it would be possible to detect 16M and 2308 CFU, if present, in a

serum sample using DNA isolation and conventional PCR using the Omp25 and Baily

primers.

B. abortus 2308 and B. melitensis 16M in Urine

The PCR assays for urine spiked with 2308 and 16M CFU provided results

similar to those for 2308 and 16M CFU dilutions in sterile H_2O. Specifically, DNA

isolations performed on 2308 and 16M CFU diluted in urine provided positive banding in

every dilution ($1x10^9$-$1x10^1$ original CFU concentration) using both primer sets (Figure

18). Using urine spiked with 2308 and 16M CFU as direct template for PCR resulted in

positive visual identification up to the $1x10^5$ original CFU dilution (1×10^3 CFU/μl) using

both primer sets, which is identical to the results observed using 2308 and 16M CFU

diluted in sterile H_2O (Figure 19).DNA isolations performed using urine dilutions of

2308 and 16M provided complete detection, with banding in every dilution across both

primer sets. With these results, it would be possible to detect *Brucella* CFU in urine, if

present, using the Omp25 and Baily primers and conventional PCR techniques following

the isolation of DNA from the sample.

Discussion

This is the first known study to investigate the limit of detection when testing

bodily fluids for the presence of *Brucella* cells using conventional PCR. It is unknown

Omp25   Baily

16M

2308

Figure 16.

Agarose gel electrophoresis of the PCR products of 16M and 2308 CFU dilutions in serum using the Omp25 and Baily primer sets. This limit of detection was consistent across both species and primer sets.

The order of the lanes is as follows:

> 1. 1kb standard size marker
> 2. Negative control
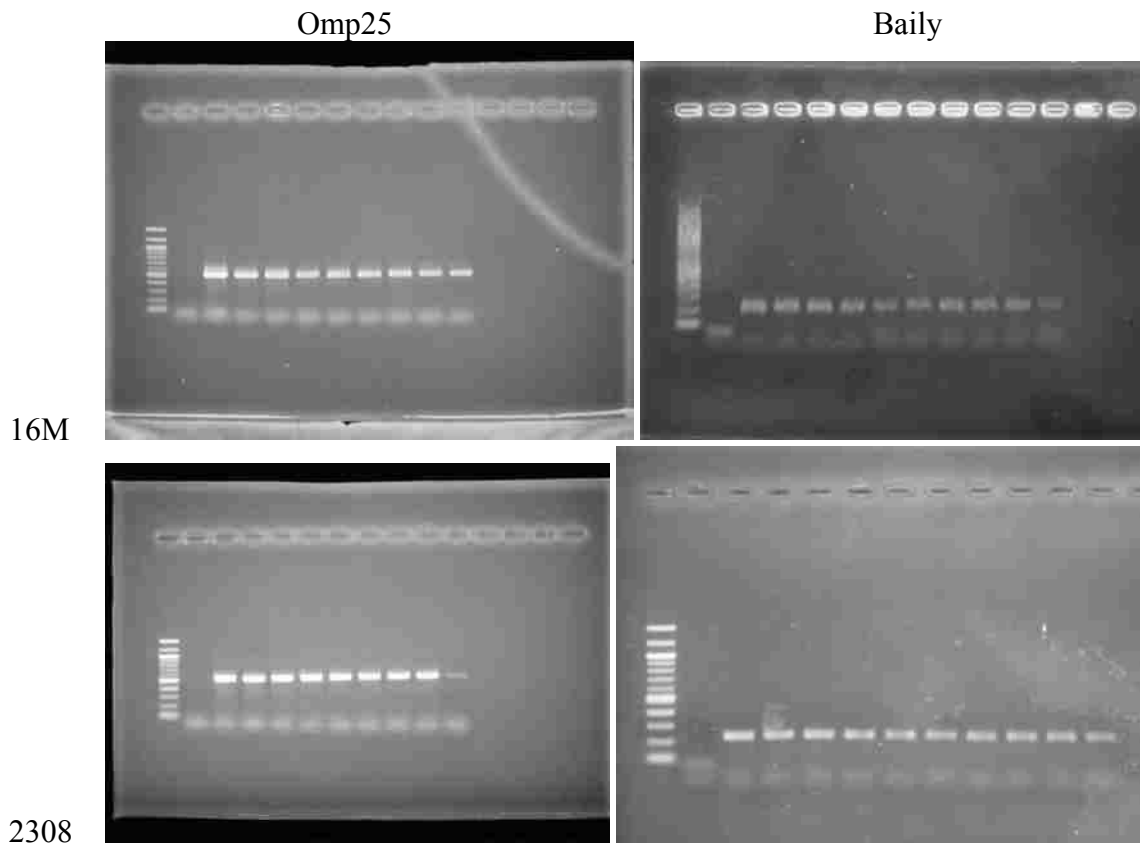> 3-12. CFU concentrations from 10^8 CFU/μl – 1 CFU/10 μl
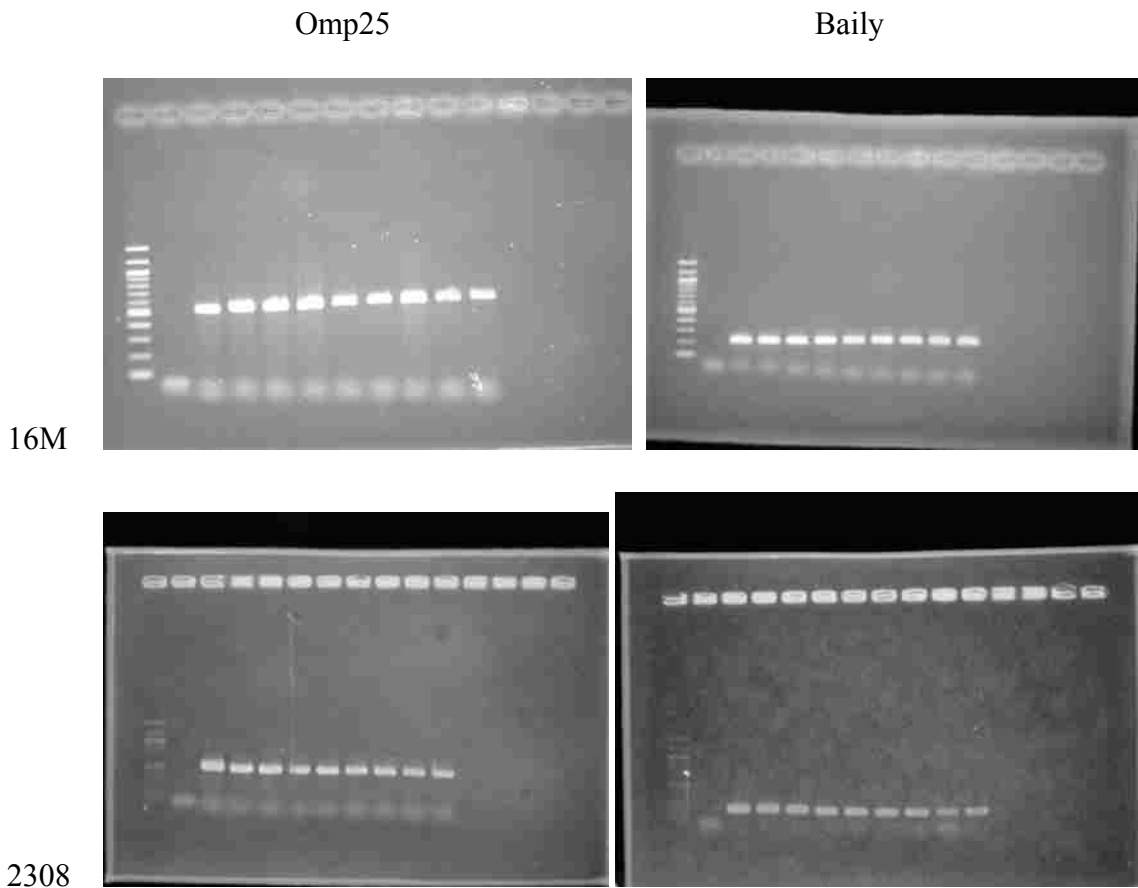
Figure 17.

Agarose gel electrophoresis of the PCR products of DNA isolated from dilutions of 16M and 2308 CFU in serum using the Baily and Omp25 primer sets. Detection is possible in every dilution.

The order of the lanes for every gel is as follows:

1. 1kb standard weight marker
2. Negative control
3-11. DNA isolation concentrations from 10^7 CFU/μl – 1 CFU/10μl

Omp25   Baily

16M
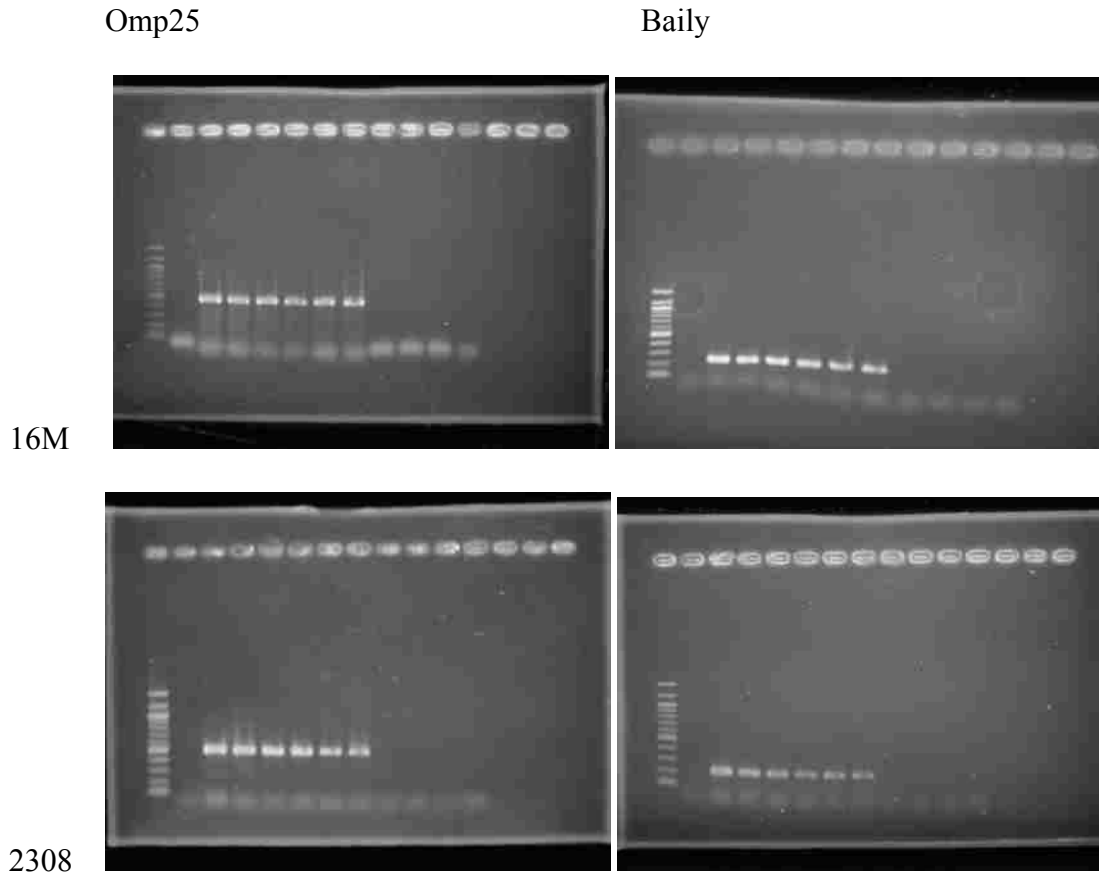
2308

Figure 18.

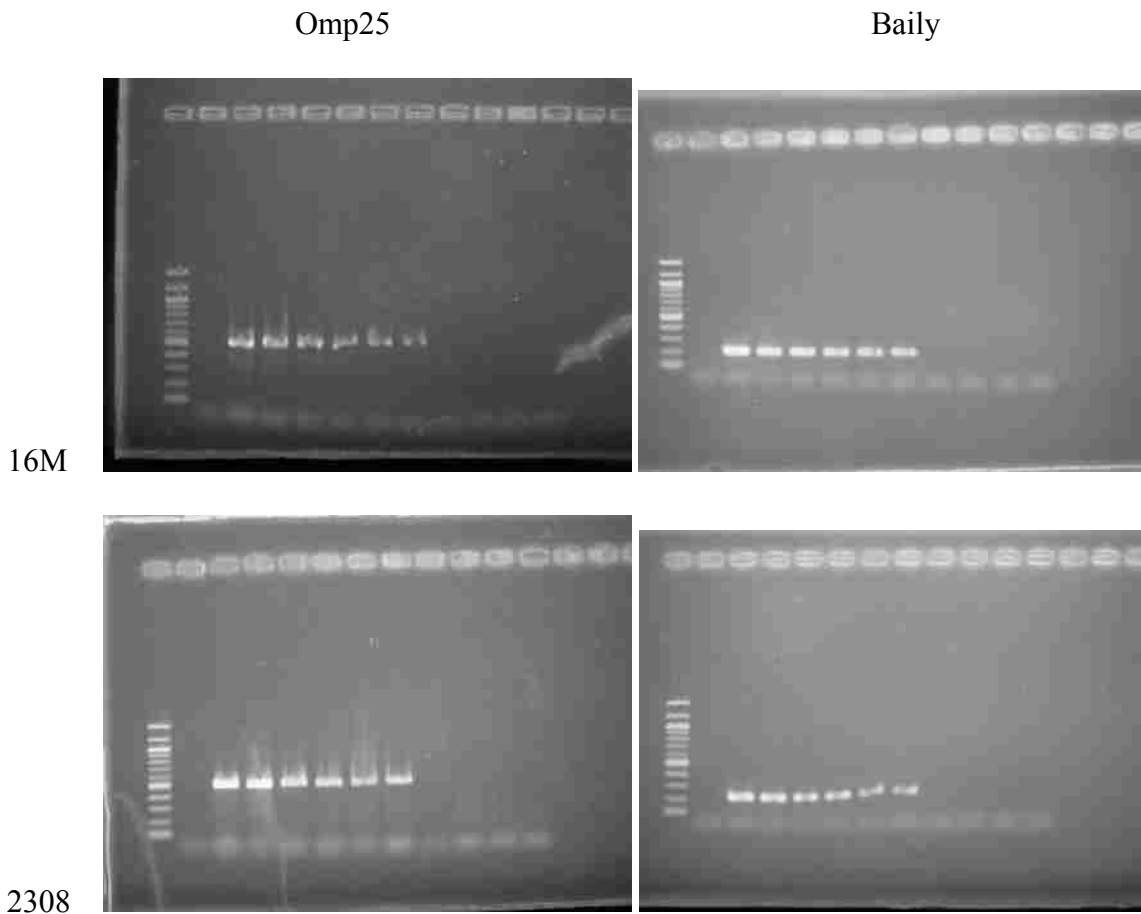Agarose gel electrophoresis of the PCR products of 16M and 2308 CFU dilutions in urine using the Baily and Omp25 primer sets. This limit of detection was consistent across both species and primer sets.

The order of the lanes for each gel is as follows:

        1. 1kb standard weight marker
        2. Negative control
        3-12. DNA isolation concentrations from 10^8 CFU/µl – 1 CFU/10µl

Omp25   Baily

16M

2308

Figure 19.

Agarose gel electrophoresis of the PCR products of DNA isolated from dilutions of 16M and 2308 CFU in urine using the Omp25 and Baily primer sets. Detection is possible in every dilution.

The order of the lanes is as follows:

      1. 1kb standard size marker
      2. Negative control
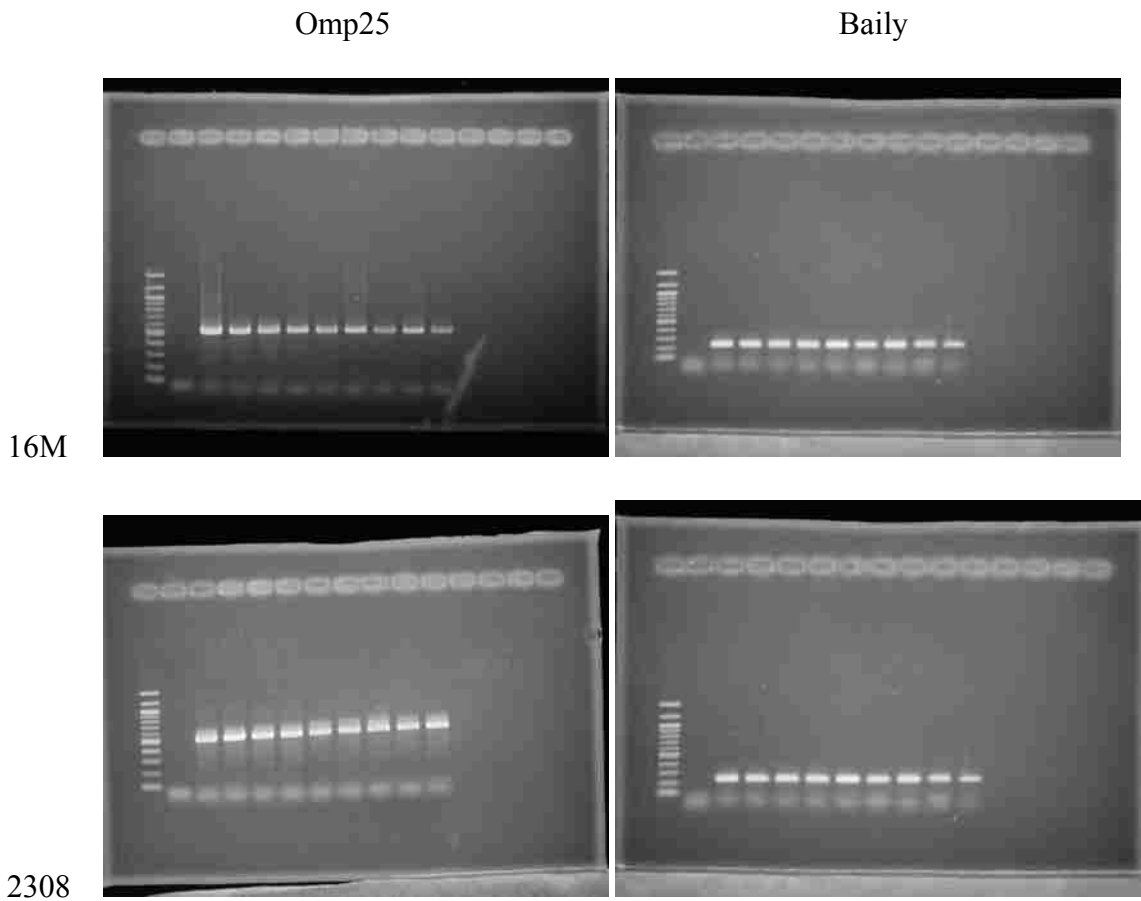   3-11. 16 M CFU concentrations from 10^7 CFU/μl – 1 CFU/10 μl

what the content of bacteria is in an actual case sample at different stages of infection, and further investigation would be needed to examine these quantities.

The results of each bodily fluid assay were compared to a control study performed in sterile H_2O using both *B. melitensis* 16M and *B. abortus* 2308 DNA isolations and both primer sets. Detection was possible for DNA isolations from concentrations of each species from $1x10^{10}$ CFU/ ml through $1x10^1$ CFU/ ml for the PCR assays using known concentrations of cells. An assay was performed using just CFU as template for PCR (without DNA isolation) and the limit of detection for each species using each primer set was observed at $1x10^5$ CFU/ ml. For the quantified DNA isolated from an unknown concentration of cells, a limit of detection for 16M using the Omp25 primers was found to be $9.35x10^{-5}$ µg/µl and the limit of detection for 2308 using the Omp25 primers was found to be $8.72x10^{-6}$ µg/µl. For the quantified DNA isolated from an unknown concentration of cells, a limit of detection for *B. abortus* 2308 using the Baily primers was found to be $8.72x10^{-8}$ µg/µl and the limit of detection for *B. melitensis* 16M using the Baily primers was found to be $9.35x10^{-8}$ µg/µl. These preliminary findings were used to compare each of the following assays to determine the effect of each bodily fluid on DNA isolation and PCR amplification on detection capabilities using conventional PCR.

A limit of detection assay was also performed for the ORF-944 region E primers for both 16M and RB51-QAE. This assay produced results similar to those observed for the Omp25 primers for 16M and the limit of detection for RB51-QAE ($4.0x10^{-6}$ µg/µl) was similar to the limit of detection for 2308 using the Omp25 primers ($8.72x10^{-6}$ µg/µl). Based on these findings, it can be assumed that the detection of RB51-QAE using the

ORF-944 primer set may be comparable to the limit of detection findings for 2308 using the Omp25 primer set in the following experiments.

The optimization of thermal-cycler protocols has been a standard procedure for many preliminary PCR assays, especially those using new primer sets. The addition of 5 cycles to each protocol increased amplification to a degree that improved detection capabilities significantly. The following results are based on the elongated thermal-cycler protocol.

Whole blood is used for culturing assays, and for this reason was the first fluid tested in this study. It is easy to obtain through non-invasive procedures from humans or animals, and animal slaughter is not required. However, there are several drawbacks to using whole blood for PCR amplification of pathogen DNA. First, the erythrocytes present in a whole blood sample interfere with standard DNA isolation techniques using a spin column. The RBCs present in the sample can clog the spin column, inhibiting efficient collection and reducing the amount of DNA present in the final isolate volume. Second, some components of whole blood, such as hemoglobin, can inhibit amplification by PCR.

Whole blood was not found to be an optimal candidate for detection of *Brucella* in a blood sample. Using CFU of *B. abortus* 2308 and *B. melitensis* 16M in whole blood as template for PCR amplification was not detectable using neither primer set in any of the diliutions tested. For DNA isolated from whole blood dilutions, the limit of detection was found at $1x10^3$ CFU/ ml for 2308 using both primer sets and for 16M using the Baily primers. The limit of detection for B. melitensis 16M using the Omp25 primers yielded a

limit of detection at 1×10^4 CFU/ ml. All results for DNA isolated from whole blood dilutions show decreased detection capability when compared to controls.

The differences between procedure 1 and procedure 2 for DNA isolation from whole blood provided an insight into the limitations of the Qiagen kit used. The main difference between the two is that the lab procedure generally used employed a greater number of cell lysis buffers for a greater period of time. Furthermore, a larger sample could be examined using the laboratory technique and thus, a greater final volume of *Brucella*-specific DNA could be obtained for study.

Plasma was chosen for investigation because plasma samples contain the buffy coat, which is a layer of white cells found between the erythrocytes and plasma layers in an anti-coagulation collection tube after settling of whole blood. Because *Brucella* are sequestered into the macrophages for replication, studying this layer in plasma is of importance. Plasma is a more fluid substance that generally does not interfere with DNA isolation techniques. As with blood, it is also easy to obtain through non-invasive procedures. Detection of *B. melitensis* 16M CFU was decreased to 1×10^7 CFU/ ml in plasma when compared to controls. Although there still remain PCR-inhibiting factors in plasma, there are far fewer than when compared to whole blood. Detection of *Brucella* CFU in plasma was similar to controls and detection of DNA isolated from plasma dilutions provided detection in all dilutions studied across both species and primer sets. This provides evidence that plasma is an acceptable fluid for the use of *Brucella* cellular detection when subjected to DNA isolation.

Serum has been tested widely in PCR investigations and thus, has been examined here. The issue with using serum to diagnose brucellosis is that bacteria are largely

absent from the serum following the bacteremic stage of infection. However, serum does not appear to inhibit DNA isolation and the PCR-inhibitors found within this substance are minimal. The assay exploring the detection capabilities of the Omp25 and Baily primers of 16M and 2308 in serum produced results similar to those seen in the control group and in the plasma group. Specifically, a limit of detection was observed at 1×10^3 CFU/μl for CFU and detection of isolated DNA was positive throughout every dilution for both species of *Brucella* using both primer sets. This suggests that serum has minimal inhibitions to DNA isolation and PCR amplification and it would be possible to detect *Brucella* cells, if present, in a serum sample using conventional PCR.

Urine, even when concentrated, appeared to have the least inhibiting effects in both DNA isolation and PCR, although crystal in urine have been shown to be inhibitive to PCR in the past. The assays performed with urine dilutions provided results similar to controls. DNA isolation provided complete detection in every dilution across both species and primer sets. Urine has been examined in previous studies for the detection of *Brucella* species by PCR. However, only two species of *Brucella* are shed in the urine and neither is of any real threat to humans. Nevertheless, practical applications may be potentially applied in the diagnosis of animals.

` Ultimately, plasma, subjected to DNA isolation, is the best candidate for detection of *Brucella* cells from a blood sample. Whole blood was found to be inhibitive of both DNA isolation and PCR amplification. *Brucella* cells may not be present in the serum following bacteremia and thus, there may be no CFU present for detection using PCR at various stages of infection. Urine was the most efficient fluid in PCR assays and provided results most similar to the control groups, but may be unconventional for

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practical applications. Furthermore, plasma contains the buffy coat layer, where *Brucella* cells may reside within macrophages. DNA isolation provided superior detection when compared to using CFU as template for all groups tested. Although this procedure takes longer and requires more cost than using CFU alone, using CFU as template is not a feasible method of accurate detection. Another factor to consider is the stage of infection at which the blood sample is taken. For example, detection may be capable in serum if taken while the subject or animal is bacteremic, otherwise *Brucella* cells may not be present for detection. Periodic testing may also be an optimal route for the positive identification of brucellosis in suspect infections.

These experiments show that in optimal conditions, it is possible to positively identify *Brucella* present in a blood or urine sample using conventional PCR as a diagnostic tool. Because this is the first series of experiments known to be performed to study the limit of detection capable when using bodily fluids, this information can be of great importance in furthering studies on detection of *Brucella* in a clinical setting using PCR. If a reliable PCR technique, using either conventional or Real-Time methods, could be implicated in endemic countries, diagnosis capabilities would be greatly improved in both animals and humans. Not only would diagnosis be performed up to four times more quickly, but the tests developed may be more sensitive as well. Further studies are needed to fully develop an optimal and reliable PCR technique for such purposes are ongoing.

SUMMARY AND CONCLUSIONS

To better understand the role of a pathogen in disease, it is important to identify and examine its virulence factors on both a genetic and protein level. Many methods have been used to evaluate antigenic determinants and virulence genes of *Brucella* species and their role in infection, including transposon and signature-tagged mutagenesis (STM) (Ocampo-Sosa et al. 2008, Pei et al. 2007, Liautard et al. 2007, Zygmunt et al. 2006, Wu et al. 2006, Kahl-McDonagh ad Ficht 2006, Lestrate et al. 2000 & 2003, Hong et al. 2000, Foulongne et al. 2000, Sangari et al. 1991, Allen et al. 1998, Smith et al. 1987), cellular immunoblotting (Brooks-Worrell et al. 1992a & 1992b), and deletion mutants (Nijskens et al. 2008, Miyoshi et al. 2007, Perry 2007, Delpino et al. 2007, Edmonds et al. 2002a 2002b 2001 & 2002, Phillips et al. 1997, Elzer et al. 1996 &1994).

Genomic mapping of several *Brucella* species has aided in the exploration of virulence factors and their effects both *in vivo* and *in vitro*. The genome of *B. melitensis* 16M was completely sequenced by Vito G. DelVecchio et al. in 2002 and several other strains have since been mapped from five species of *Brucella*. A putative hemagglutinin was identified in *B. melitensis* (as well as *B. ovis*, with homologous sequences found in *B. suis* and *B. canis*) that was not present in the *B. abortus* genome. The gene sequence for this putative hemagglutinin was identified as Region E and hypothesized as a significant virulence factor for *B. melitensis*.

Brucella hemagglutinins have been studied across several species and hosts and the ability of *Brucella* species to agglutinate red blood cells of several hosts has been documented (del C Rocha-Gracia 2002, Rocha et al. 1999, Zheludkov 1982, Diaz et al. 1967). In 1999 Rocha et al. described that *Brucella* strains are able to hemagglutinate

human, hamster and rabbit red blood cells (RBC). From this experiment, an unnamed 41kD surface protein was identified as a possible hemagglutinin and eukaryotic cell adhesion.   More recently, del C Rocha-Gracia identified a specific hemagglutinin (labeled SP29) in 2002 which is present on the surface of all *Brucella* strains tested. SP29 is described as a 29kD surface protein and was found to agglutinate RBC samples from human, rabbit, hamster, rat, and mouse sources.

These studies have supported the hypothesis that the region E protein has hemagglutinative properties, is expressed as a cell surface protein, and shows aspects of specificity toward a caprine host. Furthermore, it has been shown that the addition of region E to RB51 increases the survivability and colonization of RB51 in the goat model, meriting further investigation into the potential development of an improved vaccine for brucellosis. *In vivo* studies showed that the region E protein induces the production of anti-region E antibodies in the caprine host as well as an increase in the production of *Brucella*-specific antibodies following inoculation, when compared to the parental strain. PCR amplification data obtained using primers to the region E gene and the isolated DNA of RB51-QAE and 16M have suggested that it would be possible to use conventional PCR to detect species of *Brucella* that carry the region E gene sequence. The detection capabilities of the region E primers were similar to those observed for the Omp25 and Baily primers designed to detect all species of *Brucella.* Although not confirmed, it is suggested at the region E primers, having performed similarly in control assays, would also be effective in detecting region E-encoding *Brucella* species in bodily fluids. The results of conventional PCR assays using *Brucella* cells diluted in various bodily fluids supported the hypothesis that it is possible to detect the presence of *Brucella*

CFU in bodily fluids using conventional PCR. Additional research would be needed to assess the full potential of using the region E, Omp25, and Baily primers to successfully detect *Brucella* cells in bodily fluids for diagnostic purposes. These results merit further research into both the potential role of region E in improving the efficacy of the RB51 vaccine, and the use of conventional PCR as a diagnostic tool for the purposes of strengthening brucellosis eradication efforts worldwide.

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