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# Brucella melitensis: the evaluation of a putative hemagglutinin gene's effect on virulence in the caprine model

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# *BRUCELLA MELITENSIS*: THE EVALUATION OF A PUTATIVE HEMAGGLUTININ GENE'S EFFECT ON VIRULENCE IN THE CAPRINE MODEL

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**DEDICATION**

*"Father, I stretch my hands to Thee*
*No other help I know;*
*If Thou withdraw Thyself from me,*
*Oh! Whither shall I go?"*
*~An excerpt from a hymn by Charles Wesley*

For my mother,
Barbara Jean Johnson Perry

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*"Trust in the LORD with all thine heart; and lean not unto thine own understanding. In all thy ways acknowledge Him, and He shall direct thy paths."*
*~Proverbs 3:5&6*

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## ABSTRACT

*Brucella melitensis* is a facultative intracellular bacterial pathogen that causes abortions in goats and sheep and Malta fever in humans. The zoonotic disease brucellosis causes severe economic losses in the Mediterranean region and parts of Africa, Asia, and Latin America.

With the completion of the genomic sequences of *B. abortus* 2308 and *B. melitensis* 16M, no classical virulence factors were found; and the chromosomes were virtually identical. However, in *B. melitensis*, a putative hemagglutinin gene was identified which is absent in *B. abortus*. The possibility of the hemagglutinin gene being a potential virulence factor was evaluated via gene replacement/deletion in *B. melitensis* and expression *in trans* in *B. abortus*.

The hemagglutinin gene was PCR-amplified, cloned into pBBR1MCS-4, and electroporated into *B. abortus* 2308 yielding *B. abortus* 2308-QAE. A kanamycin-Region E-kanamycin disrupted gene fragment (KAN-E-KAN) was also generated and electroporated into *B. melitensis* 16M. The resulting mutants were characterized biochemically to confirm its *Brucella* origin and screened by antibiotic selective pressure.

A colonization study of non-pregnant goats infected with *B. abortus* 2308, *B. melitensis* 16M, *B. abortus* 2308-QAE, or *B. melitensis* 16MΔE revealed no attenuation of the 16MΔE mutant when compared to 16M at 4, 7, and 21 days post inoculation. The study also showed that both the variant and the mutant were capable of infecting and disseminating throughout the host.

All four strains were introduced into the pregnant goat model and evaluated for pathogenicity. Pregnancy/delivery results revealed 27%, 78%, 67%, and 50% abortion

rates in goats infected with 2308, 16M, 2308-QAE, and 16MΔE, respectively. Bacterial culture of tissues from 2308, 16M, 2308-QAE, 16MΔE -exposed goats revealed 45 %, 79%, 75%, and 100% colonization of dam/kid pairs, respectively. The expression of the *B. melitensis* 16M hemagglutinin gene *in trans* in 2308-QAE revealed a significant ($p \leq 0.05$) increase in colonization and abortion rates when compared to *B. abortus* 2308, mimicking the virulence of *B. melitensis* 16M in pregnant goats. The *B. melitensis* 16MΔE disruption mutant colonization and abortion rates demonstrated no attenuation in colonization but did show a 28% reduction in abortions when compared to *B. melitensis* 16M**.**

# INTRODUCTION

Over 100 years after Lieutenant Colonel David Bruce, a physician in the Royal Army, discovered "*Micrococcus melitensis*" in the spleens of British soldiers residing on the island of Malta in the Mediterranean Sea (Moreno and Moriyon 2002), Del Vecchio et al (2002a) presented the genomic sequence of *Brucella melitensis*, which was renamed after Dr. Bruce (Spink 1956). This revelation brought new insight into the biology and pathology of the organism. *Brucella* species are short, non-motile, non-sporulating, non-encapsulated, Gram-negative aerobic rods. They are facultative intracellular pathogens of animals and humans (Corbel 1997). The genus *Brucella* consists of six species, each with a preference for a primary host and varying degrees of pathogenicity. *B. melitensis* primarily infects goats and is the most pathogenic for humans. *B. abortus* infects cattle; *B. canis* causes infection in dogs; *B. ovis* is pathogenic for sheep; *B. suis* infects pigs; and *B. neotomae* causes infection in the desert wood rat. In recent years, there have also been *Brucella* species isolated from marine mammals, mostly seals and cetaceans (Bricker et al 2000).

Brucellosis, the zoonotic disease that is caused by these organisms induces abortions in wild and domestic animals and Malta or undulate fever in humans (Dalrymple-Champneys 1960). The disease is transmitted through the consumption of non-pasteurized milk and milk products or by direct contact with diseased animals or animal carcasses. The organism penetrates the skin or mucus membranes and travels to the lymph nodes, which become hemorrhagic, leading to bacteremia and the dissemination of the bacteria throughout the body (Elzer 2002).

The spread of brucellosis has been essentially controlled in developed countries, but the disease still poses a threat in the Mediterranean region, parts of Asia, Africa, and Latin America. The most recent concern focuses on the potential use of *Brucella* species, primarily *B. melitensis*, as an agent of biological warfare because of the debilitating disease it causes. Widespread dispersal of aerosolized *B. melitensis* would pose a biological, agricultural, as well as an economical threat to all countries involved.

The *Brucella* genus is highly homogeneous with all members showing greater than 90% homology in DNA-DNA pairing studies (Verger et al 1985; Vizcaino et al 2000), and little is known about *Brucella* virulence. This study evaluated the virulence of a putative ~2.0 kilobase (kb) hemagglutinin gene, Region E, using the completed genome of *B. melitensis* (Del Vecchio et al 2002a). Comparison of the chromosomes of other *Brucella* species with that of *B. melitensis* 16M revealed the absence of this hemagglutinin gene in *B. abortus* 2803. Experiments using a Region E disruption mutant of *B. melitensis* 16M and a variant of B. *abortus* 2308 expressing Region E *in trans* were carried out in the caprine model to provide insight into molecular basis of *Brucella* virulence and possible vaccine development.

## LITERATURE REVIEW

Brucellosis is an infectious disease caused by bacteria of the genus *Brucella*. These bacteria are primarily passed among animals causing disease in many different vertebrates. Various *Brucella* species infect sheep, goats, cattle, deer, elk, pigs, dogs, and other animals. Humans become infected by coming in contact with animals or animal products that are contaminated with these bacteria. *Brucella melitensis*, *B. abortus*, and *B. suis* are zoonotic pathogens which can infect humans. *B. canis* may cause infections in immunosuppressed individuals (Young 2000). Brucellosis can cause a range of symptoms in humans that are similar to the flu and may include fever, sweats, headaches, arthritis, back pains, and physical weakness. Severe infections of the central nervous systems or lining of the heart may also occur resulting in meningitis, spondylitis, endocarditis, or even death. Brucellosis can also cause long-lasting or chronic symptoms that include recurrent fevers, joint pain, and fatigue. Bacteriologic culture and serological agglutination tests are used for the diagnosis of infected humans and animals.

Brucellosis is not very common in the United States in that 100 to 200 human cases occur each year. However, brucellosis can be very common in countries where animal disease control programs have not reduced the amount of disease among animals. Although it can be found worldwide, brucellosis is more common in countries that do not have standardized and effective public health and domestic animal health programs.

Because of its epidemic potential, the absence of a human vaccine, the drawbacks of current vaccine strains in terms of safety, and the effectiveness of aerosol infection, this agent has been classified as a biosafety level 3 pathogen and is considered to be a potential bioterrorism agent (Kaufmann et al 1997).

**Genus *Brucella***

Brucellae are very small, Gram-negative coccobacilli that may appear in pairs, short chains, or groups (Corbel and Morgan 1984). Brucellae are fastidious organisms which usually grow in nutrient-rich media within 48-72 hours of incubation at 37ºC in a 5% $CO_2$ atmosphere. They are the causative agent of the zoonotic disease brucellosis. The organisms are aerobic, non-encapsulated, and catalase and oxidase positive. They do not ferment carbohydrates and have variable urease activity (Young 1995). Based on DNA homology, it has been proposed that all six members of the genus are actually serovars of a single species (Halling et al 2005). Four members of the genus *Brucella*, *B. abortus*, *B. suis*, *B. canis*, and especially *B. melitensis* are able to cause infection in humans (Young 1995).

Brucellae are capable of evading host defense mechanisms, surviving as intracellular organisms, and are able to cause prolonged morbidity, relapses, and long-term abnormal conditions. Brucellosis is a systemic infection that may affect any organ system in the body (Shehabi et al 1990; Yagupsky 1997). Because of the wide spectrum of its clinical manifestations, brucellosis may mimic other infectious and noninfectious conditions, frequently delaying the diagnosis of the disease or even missing the disease all together (Solera et al 1997; Young 1995).

Brucellosis continues to affect large human populations living in rural areas in Mediterranean Basin (Portugal, Spain, Southern France, Italy, Greece, Turkey, and North Africa), South and Central America, Eastern Europe, Asia, Africa, the Caribbean, the Middle East, and Latin American countries where the organisms are endemic (Arnow et al 1984; Gotuzzo et al 1986; Shehabi et al 1990; Yagupsky 1994; Solera et al 1997). As a

result of infection control measures, the incidence of human brucellosis has declined over the past fifty years in developed countries. Most cases in these countries represent occupational disease, travel-acquired infections, or accidental laboratory exposure.

***Brucella abortus***

*Brucella abortus*, initially isolated as *Bacillus abortus* by Bang in 1897 and eventually renamed in 1920, is the etiological agent of bovine brucellosis, an infection that leads to spontaneous abortion, premature calving, and infertility in cattle. The fetus, placenta, and uterine fluid typically contain large quantities of *B. abortus* bacteria, which can infect other cattle coming into contact with an infected animal and its vaginal secretions around the time of calving. The organism is also excreted in the milk.

Brucellosis is contracted by humans by drinking infected unpasteurized milk or from contact with discharges from cattle or goats that abort their fetuses. This disease does not spread from person to person. Symptoms include intermittent or irregular fever of variable duration, headache, weakness, profuse sweating, chills, weight loss, and generalized malaise.

In cattle, brucellosis is primarily a disease of the female. Bulls can be infected, but they do not readily spread the disease. The organism localizes in the testicles of the bull, resulting in orchitis. In the female, the organism is prevalent in the udder, uterus, and lymph nodes adjacent to the uterus. The infected cows exhibit symptoms which may include abortion during the last trimester of pregnancy, retained afterbirth, and weak calves at birth (Enright 1990). Typically, infected cows usually abort only once; subsequent calves may be born weak or healthy. Some infected cows will not exhibit any clinical symptoms of the disease and give birth to normal calves. Millions of organisms

are shed in the afterbirth and in fluids associated with calving and aborting. The disease is spread when cattle ingest contaminated feed or lick calves or aborted fetuses from infected cattle.

This species is able to cross the species barrier affecting both other livestock and humans (Young 1995). In livestock, it causes billions of dollars in losses due to abortions in cattle. This species is also listed as a civilian, military, and agricultural bioterrorism agent.

***Brucella melitensis***

*Brucella melitensis*, the first species in the genus *Brucella* to be described, causes abortions in female goats and sheep, unilateral orchitis in males, and Malta fever in humans (Alton 1990a). Sir David Bruce, a British army surgeon, discovered the organism in 1887 as the causative agent of Mediterranean or Malta fever (Moreno and Moriyon 2002). The organism now bears his name coupled with "melitensis," which is Latin for Malta. *B. melitensis* is prevalent in Mediterranean and Middle Eastern countries through Central Asia to China and southern areas of the former Soviet Union. Some areas of Africa and India, as well as Central and South America, are also affected. This species' natural hosts may be goats and sheep, but the organism is the least species-specific of the brucellae (Alton 1990a).

Sheep and goats and their products are the main source of infection, but *B. melitensis* in cattle has emerged as an important problem in some southern European countries, Israel, Kuwait, and Saudi Arabia. The disease in goats resembles the disease in *B. abortus*-infected cattle (Enright 1990). *B. melitensis* infection is particularly problematic because *B. abortus* vaccines do not protect effectively against *B. melitensis*

infection. Thus, bovine *B. melitensis* infection is emerging as an increasingly serious public health problem in some countries with the spread of the disease through unpasteurized dairy products received from these infected cows.

*B. melitensis* is highly pathogenic for humans, making it one of the most serious zoonoses in the world. The most prominent symptoms are weakness and intermittent fever. The disease persists for months if left untreated but is seldom fatal in humans. Infection is normally by inhalation and via abraded skin, and transmission between species occurs readily. Humans usually become infected by ingestion of contaminated, unpasteurized milk and milk products. So far, a vaccine has not yet been discovered to fight the disease in humans, and animal vaccines are pathogenic to humans.

*Brucella melitensis* is considered a potential agent for biological warfare by the US Department of Health and Human Services Centers for Disease Control and Prevention. Although rarely fatal for humans, brucellosis is highly contagious, difficult to treat, and easily transmitted, making it ideal for use in bioterrorism.

***Brucella suis***

Brucellosis caused by *B. suis* was first described by J. Traum in 1914 in swine herds in Indiana. It was initially thought to be a pathogenic *B. abortus* but was later named *B. suis* by I. F. Huddleson (Alton 1990b). Comparison of the closely-related *B. suis* and *B. melitensis* genomes revealed a set of genomic variations that could be responsible for the differences in virulence and host preference between these organisms (Paulsen et al 2002).

Domestic and feral swine are natural hosts of *B. suis* (Norton and Thomas 1976; Becker et al 1978; Zygmont et al 1982). In sows, abortion is the primary indicator of

disease, which occurs at any stage of the pregnancy. An infected sow may deliver some healthy live piglets and have some born dead or die shortly after birth. Mastitis may also be observed. In boars, there may be brucellae present in the semen without any visual indications of disease. There may also be unilateral swelling and atrophy of the epididymes and testes usually resulting in infertility. Reports of lameness; swollen joints, bursae, and tendons; and paralysis because of abscess formation near the spine have also been documented (Alton 1990b). Brucellosis caused by *B. suis* is considered to be a venereal disease with the infected boar passing the disease on to uninfected sows (Alton 1990b).

Contraction of the human disease is primarily limited to the occupational hazards of farmers and abattoir workers. Considered a potential bio-weapon, *B. suis* was the first pathogenic organism weaponised by the U.S. military during the 1950s. It is seen as a potential bioterrorism threat that could be targeted against military personnel, civilians, or food supplies (Paulsen et al 2002).

***Brucella ovis***

A rough form of *Brucella*, *B. ovis* is the primary cause of brucellosis in sheep. In its rough form, brucellae lack the hydrophilic O-polysaccharide (OPS) side chain of the lipopolysaccharide (LPS) of their outer cell membrane, differing from the typically smooth forms of *B. abortus*, *B. melitensis*, and *B. suis* (Myers et al 1972). *B. ovis* was first isolated in New Zealand by McFarlane et al and in Australia by Simmons and Hall (Blasco 1990). It has also been found in the US, Mexico, Canada, South Africa and parts of Asia, Europe and South America (Blasco 1990).

*B. ovis* may be transmitted venereally via an infected ewe. It can also be passed

from one ram to another ram by direct contact, sodomy, sharing of pens, or through shearing wounds (Blasco 1990). Ewes rarely show symptoms and only a small percentage of them actually abort (Grillo et al 1999). Abortions typically occur in cases where the ewe is exposed to the organism at early or mid-pregnancy, yet females rarely abort if they are infected before mating or late in pregnancy. However, some ewes may develop placentitis as a result of exposure to the organism which may result in weak lambs (Theon and Enright 1986). In sexually-mature rams, *B. ovis* causes epididymitis, orchitis, and infertility. Not all rams appear to be shedders of the infection. It is thought that only 40% of rams with low titer reactions shed the organism, whereas, 100% of high titer reactor rams are thought to shed the microbes (West et al 2002).

***Brucella canis***

The canine *Brucella* was first recognized in the late 1960s as a cause of abortions and reproductive failures, and it has since been recognized in several countries (Carmichael 1990). It is especially common in Mexico, Central and South America and in the southern states of the United States. It has been diagnosed in commercial or research breeding beagle kennels in several other countries, including Japan and more recently in The People's Republic of China. The disease has been reported sporadically in Europe (Wanke 2004). Humans may be infected; however, dogs and other canine species are believed to be the only true hosts. Canine brucellosis does not typically end in an animal's death, but an animal's reproductive failures can be economically detrimental to their owners (Hollett 2006).

Brucellosis in dogs is caused by the sexual transmission of *B. canis* through the mating of infected males and females. In the female dog, *B. canis* lives in the vaginal and

uterine tissues and is secreted for years and usually for life. The infected female may appear healthy with no signs of disease or indication that she is a carrier of the organisms. She can spread the bacteria to other animals through her urine, aborted fetuses, or most commonly through the act of breeding. The bacteria also infect the developing fetuses causing illness (Carmichael and Joubert 1988). Clinical signs are primarily associated with the reproductive tract. In females, the most prominent sign is abortion after 45-55 days of gestation in about 75% of the cases. These may go unnoticed, and the female may present with the complaint of "failure to conceive." In males, the bacteria live in the testicles and seminal fluids. An infected male can spread the bacteria via his urine or semen (Moore and Kakuk 1969). There are often no signs except in advanced cases where epididymitis, testicular atrophy, scrotal dermatitis, and infertility may be observed (Carmichael 1990). Semen from infected males usually contains large numbers of abnormal sperm and inflammatory cells, especially during the first three months following infection. Chronically-infected males may have no sperm or reduced numbers of immature sperm. Infected males harbor organisms in the prostate gland and epididymides (Wanke 2004). Nonspecific signs in both sexes include lethargy, loss of libido, premature aging, and generalized lymph node enlargement.

Litters of infected dogs are commonly aborted, usually in the last two weeks of gestation, or the puppies may die shortly after birth. Usually, the fetuses are partially decayed and accompanied by a gray to green vaginal discharge. This discharge can contain very high numbers of *B. canis*. Early embryonic death and re-absorption or abortion 10-20 days after mating may occur in some cases. If they die early, embryos may be reabsorbed, and the female may never appear to be pregnant at all.

### *Brucella neotomae*

*B. neotomae* was isolated from the desert wood rat, *Neotoma lepida*, by Stoenner and Lackman in 1957 (Cameron and Meyer 1958). It was identified as a new species of *Brucella* on the basis of conventional genus speciation, including the organism's behavior on differential dye media, $CO_2$ requirements, and $H_2S$ production. The organism was found to display a distinct difference from the three main species, *B. abortus*, *B. melitensis*, and *B. suis*, and all sub-classifications within the species (Huddleson et al 1957; Cameron and Meyer 1958).

### Marine Mammal Species

Recently, a number of *Brucella* isolates were described whose properties do not closely agree with the descriptions for recognized species. The status of most of these strains has not been finally decided, and it is possible that some or all of them will eventually be found to correspond to atypical cultures of existing species or biovars. In several reports, these new *Brucella* species were isolated from marine mammals, predominantly seals and cetaceans and an otter from Scotland and the coast around northern England and from a bottle-nosed dolphin from California (Ewalt et al 1994). Identification of these organisms was based on serology, morphology, staining, metabolic phenotype, culture characteristics, and phage typing (Vizcaino et al 2004; Clavareau et al 1998; Jahans et al 1997). Characterization of these strains failed to assign them to a known species of the genus *Brucella,* and many findings have raised questions concerning exposure, prevalence of infection, distribution and possible pathogenicity and zoonotic potential of these new species (Foster et al 1996; Ewalt et al 1994).

## Vaccines

Development of efficacious vaccines against brucellosis is at the forefront of prevention of the disease. Many experiments have been conducted using killed and live vaccine candidates (Schurig et al 2002). Killed vaccine candidates usually confer poor immunity, whereas live-attenuated vaccines of virulent strains typically provide adequate immunity against abortion but can lead to release of the pathogenic organisms and possibly expose susceptible animals to infection (Nicoletti 1990a). Ideal qualities of a vaccine candidate include: (1) long duration of immunogenesis; (2) minimum interference with diagnostic tests; (3) easy production and storage of the vaccine with long stability; and (4) minimum adverse effects in vaccinated animals with no danger to humans in the event of exposure (Nicoletti 1990a).

The three primary vaccines used to prevent brucellosis in cattle, goats and sheep include *B. abortus* strain 19 (S19), *B. abortus* strain RB-51, and *B. melitensis* strain Rev.1. There are no effective commercial vaccines available for pigs, *B. ovis*-infected sheep (Sanmartino 2005), or humans.

### *B. abortus* Strain19

Until recently, Strain19 (S19) was the only vaccine used in the brucellosis control programs for cattle in the United States. After it became the official vaccine in the US, it was subsequently used throughout the world. S19 is the most widely used vaccine for the prevention of bovine brucellosis (Nicoletti 1990b). It is like other smooth B. *abortus* strains containing an intact LPS with an O-side chain. S19 originated after a virulent *B. abortus* isolation from a cow was serially-passaged and stored at room temperature for several months and appeared to be attenuated upon replication (Sanmartino 2005).

Protection in pregnant heifers induced by S19 is reported to be 70-90% against abortion and/or infection (Sanmartino 2005, Confer et al 1985; Nicoletti 1984). The S19 vaccine was and still is an effective tool in brucellosis control. However, using the S19 vaccine has its advantages and disadvantages. It is a live vaccine that stimulates both the humoral and cell-mediated responses of the immune system of vaccinated animals to resist a challenge with virulent *Brucella* spp. which otherwise results in disease. Disadvantages include abortion, orchitis, pyrexia, anorexia, and occasional persistent udder infection (Nicoletti 1990a). Normally, a vaccinated animal retains resistance to disease for an extended period of time (years), but the detectable antibodies disappear in a few months.

Unfortunately, the serological tests used to detect brucellosis-infected cattle cannot differentiate between antibodies produced against S19 vaccine and antibodies produced against field strains due to S19's smooth orientation. If a vaccinated animal is tested too soon following vaccination or if the vaccinated animal retains the antibodies stimulated by the vaccine for an extended period, the vaccinated animal would result in a false positive. In addition, some animals with developing uteruses and/or mammary glands vaccinated with S19 vaccine may become permanently infected with the vaccine organism, constantly producing antibodies against it and continuing to test positive. If an animal is vaccinated with the S19 vaccine after the animal reaches reproductive maturity, the animal may be prone to become permanently infected with the live vaccine organism and will continue to be stimulated to produce antibodies against the vaccine.

Calves born to S19-vaccinated cows acquire anti-*Brucella* antibodies from the cow through the colostrum (first-milk) immediately after birth. These acquired antibodies normally circulate in the calves' blood system for 4-6 months and can neutralize or kill

the live vaccine organisms if the calf is vaccinated during the time it still possess the antibodies from the colostrum. The routine vaccination of cattle/herds against brucellosis is restricted to heifers between the ages of three to eight months of age (Nagy et al 1967; Poester et al 2006).

***B. abortus* RB-51**

In 1996, the USDA officially recognized and began using RB-51 as a brucellosis vaccine for protecting cattle against the disease. Like S19, RB-51 is a live vaccine derived from, *B. abortus.* After 51 serial passages of smooth *B. abortus* 2308 onto tryptic soy broth agar plates supplemented with rifampicin and penicillin, Schurig et al isolated the rough attenuated *B. abortus* Strain RB-51 (Schurig et al 1991; Schurig et al 2002). The RB-51 vaccine was derived from this rifampicin-resistant mutant of smooth *B. abortus* 2308 which lacks almost all of the LPS O-side chain. Vaccination of calves three to ten months of age with RB-51 has been evaluated to provide protection against abortion and infection statistically similar to that of S19 with no signs clinical disease, no pathogenic lesions associated with acute brucellosis, and with rapid clearance of the organism from draining lymph nodes (Cheville et al 1992; Cheville et al 1993; Cheville et al 1996). Studies have shown that vaccination of pregnant cattle with RB-51 elicits both humoral and cell-mediated immune responses without infection and/or abortion (Palmer et al 1997). Unlike S19, RB-51 vaccine does not stimulate antibodies that are detected by the standard brucellosis serological tests, alleviating the problem of having some brucellosis-vaccinated cattle test positive with standard serological tests (Cheville et al 1993; Stevens et al 1994). The RB-51 vaccine is approved for use in most brucellosis eradication programs for subcutaneous vaccination of calves four to twelve

months of age at a full dose of 1.0-3.4 x $10^{10}$ CFU (Poester et al 2006).

***B. melitensis* Rev. 1**

Rev. 1 vaccine was developed by Elberg and Herzberg in 1957 from a non-dependent reverse mutant of the virulent streptomycin-dependant strain of *B. melitensis* 5056 as a live vaccine against *B. melitensis* in goats and sheep (Sanmartino 2005). Rev. 1 is a smooth bacterium with a complete LPS which induces a similar antibody response as that caused by the field strains and can not be easily differentiated by conventional serology. The vaccine is a streptomycin-resistant *B. melitensis,* and it can become an important health problem if accidents occur involving those persons working with the vaccine (Blasco and Diaz 1993).

Subcutaneous vaccination with Rev. 1 is recommended for goats and sheep between four to six months of age. Using standard subcutaneous doses (1 x$10^9$ - 2 x$10^9$ CFU) of the vaccine, Rev. 1 yields a two to three month widespread and persistent infection of the animal by the organism, actively colonizing the spleen and several lymph nodes. It also induces a powerful and long-lasting serological response, which can interfere with diagnostic tests (Blasco 2006). In areas with widespread infection or the likelihood of re-infection, it is recommended that adult animals receive reduced doses of the vaccine (Blasco 1997). Conjunctival vaccination induces sufficient protection in animals three to six months of age and also reduces the possibility of serological interference.

Both standard and reduced doses of Rev. 1 induce abortions in sheep and goats vaccinated during pregnancy. Studies have demonstrated that even reduced doses are not entirely safe or protective (Alton 1970; Blasco 1997) against *B. melitensis* in sheep and

goats (Alton 1970). Nevertheless, conjunctival vaccination of animals with Rev.1 during breeding periods can effectively provide protection and reduce the risk of vaccine-induced abortions (Blasco 1997).

**Human Infection and Treatment**

Generally, humans are infected in one of three ways: ingestion or inhalation of *Brucella* organisms via the nasal, oral, and pharyngeal cavities; or having the bacteria enter the body through skin wounds (Brinley-Morgan and Corbel 1990). The most common way to be infected is by eating or drinking contaminated milk products. Milk from infected animals, such as sheep, goats, or cows, is contaminated with the bacteria. If the milk is not pasteurized, these bacteria can be transmitted to persons who drink the milk or eat cheeses produced from the infected animals. Inhalation of *Brucella* organisms is not a common route of infection, but it can be a significant hazard for people in certain occupations, including those working in laboratories where the organism is cultured. Inhalation of the organism is often responsible for a significant percentage of cases in abattoir employees. Contamination of skin wounds is problematic for persons working in slaughterhouses, meat packing plants, or for veterinarians. Hunters may also be infected through skin wounds or by accidentally ingesting the bacteria after cleaning killed caribou, elk, or wild pigs that were infected.

Human patients suffering from brucellosis are routinely treated with combinations of antibiotics, such as rifampin and doxycycline or streptomycin and doxycycline (Solera et al 1997). Historically, 2% of untreated *B. melitensis*-infected patients die, and *Brucella*-induced deaths are still reported (Corbel 1997). Because of the zoonotic aspects

of this infectious disease, the control of brucellosis in agricultural animals is crucial in order to eradicate human brucellosis.

The World Health Organization recommends a treatment of 600 to 900 mg rifampicin and 200 mg doxycycline daily for a minimum of six weeks for acute brucellosis in adults (World Health Organization 1986). Some research supports the claim that a combination of intramuscular streptomycin with doses of oral tetracycline provides fewer relapses (Ariza et al 1985). For treatment of the disease in children, rifampicin has been recommended as the treatment of choice with cotrimoxazole as an alternative. Both drugs are associated with a high relapse rate if they are used alone; better results are usually achieved by using them in combination (Khuri-Bulos et al 1993). Relapse rates among adults and children of 5-10% are not uncommon after the completion of antibiotic treatment (Hall 1990; Memish et al 2000).

**Pathogenicity**

*Brucella* species are intracellular pathogens that are capable of survival and replication inside host phagocytic and non-phagocytic mammalian cells, which is essential for virulence (Celli 2006). Following penetration of the mucosal epithelium, the bacteria are transported to the regional lymph nodes. The spread and multiplication of brucellae in lymph nodes, spleen, liver, bone marrow, mammary glands, and sex organs occurs via macrophages. The increase of brucellae in the host is mainly due to the organisms' ability to avoid the killing mechanisms and proliferate within macrophages. Virulent *Brucella* species not only resist killing by neutrophils following phagocytosis (Riley and Robertson 1984a and 1984b; Canning et al 1986), but they also replicate inside macrophages (Jones and Winter 1992) and nonprofessional phagocytes (Detilleux

et al 1990a). Brucellae are capable of establishing themselves in replicative phagosomes inside of host macrophages for extended intracellular survival. They also appear to be capable of withstanding exposure to reactive oxygen intermediates, acidic pH, and nutrient deprivation during their time inside the host macrophage (Celli 2006). There is evidence that smooth LPS probably plays a vital role in intracellular survival since smooth organisms tend to survive much more effectively than rough ones (Zhan and Cheers 1995; Caron et al 1994). Survival in macrophages is considered to be responsible for the establishment of chronic infections and allows the bacteria to escape the extracellular mechanisms of host defense, like complement and antibodies. Many studies have been done to evaluate the relationship between the brucellae and mammalian macrophages from cattle (Harmon et al 1988; Price et al 1990), humans (Rittig et al 2001), and mice (Watarai et al 2002; Celli et al 2003), which reveal the organisms' ability to survive and replicate within those cells.

The mechanism of invasion of non-phagocytic cells, such as placental trophoblasts, is not clearly established. Within non-phagocytic cells, brucellae tend to localize in the rough endoplasmic reticulum. Placental trophoblasts are a part of the epithelial layers of the placenta of the natural host. They serve as an important interface between the maternal and fetal circulation. At late-gestation, *Brucella* are known to replicate within the placental trophoblasts of their natural ruminant host causing, the degradation of placental integrity, infection of the fetus, and possibly abortion or the birth of weak or infected animals (Roop et al 2004). Erythritol, which may serve as a growth stimulant for brucellae, is produced in large amounts by ruminant placental trophoblasts (Enright 1990). Further experiments utilizing microscopic analysis of placental tissues

from *B. abortus*-infected cows and goats also revealed that brucellae replicate in intracellular compartments associated with the rough endoplasmic reticulum of trophoblasts, suggesting a similar intracellular environment to that inside of host macrophages (Meador and Deyoe 1989; Anderson et al 1986a and 1986b). Brucellae have also been observed to infect the non-phagocytic trophoblasts cells of pregnant ruminants *in vivo* (Anderson and Cheville 1986). They have also been observed to infect epitheloid HeLA (Pizarro-Cerda et al 1998) and fibroblast Vero (Detilleux et al 1990b and 1991) cell lines *in vitro*.

### *Brucella* Genomes

Several complete genomes of the genus *Brucella*, including *B. melitensis* 16M (De Vecchio et al 2002a), have been sequenced thereby providing an opportunity for researchers to examine new perspectives for deciphering this historical, disease-causing organism. Revelations provided by the completed genomes initiates the commencement of studies pertinent to evaluating the functional annotation of products from predicted coding sequences (De Bolle et al 2004), giving researchers insight into genes and gene functions. It also creates an opportunity to study the products produced by novel genes and to evaluate their implications for survival, replication, and virulence. Proteomic studies to define biochemical pathways associated with stress responses, host specificity, pathogenicity, virulence, and vaccine development have also expanded. Comparison of genomes and proteomes among the species has shed significant light on the *Brucella* genus (Wagner et al 2002; Mujer et al 2002; Halling et al 2005; Eschenbrenner et al 2006; Ding et al 2006).

**Genetic Manipulation of *Brucella* Species**

Genomic characterizations have also enabled researchers to manipulate many *Brucella* genomes in an effort to determine gene functions, particularly those genes involved with virulence. Genetic manipulation of the genomes has become an important tool in unraveling the pathogenicity of the genus.

Although not naturally competent, brucellae are capable of accepting plasmid DNA via electroporation or after chemical treatment (Lai et al 1990; Elzer et al 1994). The broad-host-range vector pBBR1MCS is able to provide genetic complementation for all *Brucella* species (Kovach et al 1994; Elzer et al. 1995). Derivatives of pBBR1MCS have also been produced, each with a selectable marker, such as the green fluorescent protein or an antibiotic cassette, for easy identification of successful transformations and variant strains. The vector and its derivatives are not actually incorporated into the genome but are maintained at a low copy number throughout the *Brucella* genus (Elzer et al. 1995).

Additional vectors are also useful in these experiments. Suicide vectors, like ColE1-derived plasmids, containing fragments of *Brucella* DNA are unable to replicate in *Brucella* species and are not maintained within the cell (Elzer et al 1995). In many gene replacement studies, homologous recombination occurs between the suicide vector and the *Brucella* genome, resulting in successful replacement of the target gene (Ugalde et al 2000; Alvarez-Martinez et al 2001; Edmonds et al 2002a and 2002b).

Creation of *in vivo* gene mutations has become an important component in the quest to identify *Brucella* virulence factors. Genetic manipulation of *Brucella* genomes using gene replacement by homologous recombination is well documented (Lai et al.

1990; Halling et al 1991; Tatum et al 1992; Elzer et al 1994; Phillips et al 1994; Drazek et al 1995; Elzer et al 1995; Phillips et al 1995; Kohler et al 1999; O'Callaghan et al 1999; Alvarez-Martinez et al 2001; Edmonds et al 2001; Boschiroli et al 2002; Edmonds et al 2002a and 2002b).

Gene disruption via deletion or insertion mutagenesis is a technique routinely used in the process of gene replacement. Initially, production of such a gene knockout mutant involves cloning the gene of interest into an appropriate plasmid vector. This gene of interest is then mutated, *in vitro*, by deleting a portion of the gene or introducing a selectable marker, like an antibiotic resistance gene into an endonuclease restriction site in the gene (Madigan et al 2000; Edmonds et al 2002a and 2002b). The mutation in the gene of interest is then confirmed, and the plasmid is introduced, usually via electroporation, into *Brucella* species. A double crossover event within the cell replaces the wildtype gene via homologous recombination resulting in a loss of function in the gene of interest.

**Bacterial Hemagglutinins**

Bacterial hemagglutinins became interesting in the early 1900s when Kraus and Ludwig observed that some bacteria, historically staphylococci and vibrio, use substances to cause red blood cells (RBCs) to agglutinate (Netter et al 1954). A resurgence in the study of hemagglutinins occurred with the evaluations of viral hemagglutinins.
G. K. Hurst studied the effect of influenza hemagglutinins on RBCs via the allantoic fluid of chick embryos infected with the virus (Hirst 1941).

Classically, there are two types of bacterial hemagglutination: direct and indirect (Neter et al 1954). Direct hemagglutination occurs when bacteria cause the clumping of

RBCs. In indirect hemagglutination, bacteria change the RBCs, making them susceptible to agglutination by antibodies. Many bacterial antigenic components may be absorbed by RBCs, resulting in the agglutination of these modified RBCs by homologous bacterial antibodies (Neter et al 1954). As studied in viruses, hemagglutinins allow the recognition of the target cells' sialic acid-containing receptors and help facilitate the entry of the virus into the target cell via the fusion of the host endosomal and viral membranes (White et al 1997).

*Brucella* LPS has important cell surface properties yet there is no evidence showing its role in invasion (Aragon et al 1996). Varied virulence between smooth and rough strains of *Brucella* suggests that the O-side chain of LPS is not the organisms' only virulence factor. Other outer membrane proteins may also play a role in the organisms' virulence (del C. Rocha-Gracia et al 2002; Corbel and Brinley-Morgan 1984). An organism's ability to adhere to a mucosal surface is a crucial first step in the pathogenesis of many pathogens (Finlay and Falkow 1997). For that reason, brucellae entry into phagocytic and non-phagocytic cells must be preceded by direct contact of the bacteria with host target cells on the mucosal surface (del C. Rocha-Gracia et al 2002). This initial attachment of the brucellae to epithelial or RBCs is mostly unknown. Historically, *Brucella* cell components specific for cell adhesion and invasion have not been characterized, and attempts to detect invasin genes homologous to those of enterobacteria have failed (Corbel 1997). With the completion of entire *Brucella* species genomes, specifically *B. melitensis* 16M, studies have been and are currently being done to detect and characterize novel genes that may be involved in *Brucella* pathogenicity (Del Vecchio et al 2002a and 2002b). Of particular note is a putative hemagglutinin gene

found within the *B. melitensis* genome that is absent in *B. abortus* (DelVecchio et al 2002b and 2002c). The gene is present in *B. suis* and *B. canis* but with minor nucleotide substitutions. There are two copies of the gene in *B. ovis*. A NCBI Nucleotide Blast of the sequence reveals that the gene corresponds to a *B. melitensis* 16M hemagglutinin (GenBank GI 17989062). There is also some homology to a cell wall surface protein of *B. suis* 1330 (GenBank GI 23500299) (Paulsen et al 2002).

A study done by del C. Rocha-Gracia et al (2002) explored the possibility of hemagglutinins on the cell surface of brucellae serving as adhesins to eukaryotic cells through the ability of *B. abortus* and *B. melitensis* to hemagglutinate human and animal (rabbit, hamster, guinea pig, rat, mouse, sheep, and dog) erythrocytes and attempted to identify a receptor moiety involved in that reaction. The study utilized the hemagglutination test (HA), which is frequently used to detect and characterize bacterial lectin-like adhesins (Evans et al 1980; Qadri et al 1994). All *Brucella* strains (*B. abortus* 2308, *B. abortus* S19, *B. abortus* 02, and *B. melitensis* 03) tested showed hemagglutination to the RBCs from the various sources, with *B. melitensis* 03 showing the highest hemagglutination titers against all RBCs and *B. abortus* 2308 the lowest titer (del C. Rocha-Gracia et al 2002). Further studies such as these serve as evidence of the presence of hemagglutinins on the cell surface of *Brucella* species.

**Brucellosis in the Caprine Model**

Use of the goat model for the study of ruminant brucellosis has been well documented (Anderson et al 1986a and 1986b; Meador and Deyoe 1986; Meador et al 1988; Meador et al 1989a and 1989b; Elzer et al 1996; Phillip et al 1997; Roop et al 2001; Elzer et al 2002; Gee et al 2004; Zygmunt et al 2006; Kahl-McDonagh et al 2006).

The lower cost of these small ruminants, the greater quantity of animals in the available space to increase statistical significance, and the shorter gestation periods allowing for timely results are the primary advantages of using the caprine model system for the study of ruminant brucellosis (Elzer et al 2002).

Elzer et al (2002) established a goat model system to test potential vaccine candidates as well as evaluate virulence factors in knockout and disruption mutants. The system is based upon the colonization of the non-pregnant female, colonization of the pregnant female and fetus/kid, and the delivery status of the fetus/kid following exposure to the experimental pathogen. This system also allows researchers to assess a mutant strain's ability to invade a mucosal site if administered conjunctively. Survival, replication, and dissemination of the mutant throughout the body is assessed using the colonization assay. The organism's ability to colonize the dam and the fetus/kid can be evaluated through the pathogenesis assay (Elzer et al 2002).

***B. abortus***

Although goats are not the primary hosts, *B. abortus* produces similar clinical and serological results in the goat as those seen in cattle (Anderson et al 1986a and 1986b; Meador et al 1988 and 1989). The use of the caprine model to evaluate *B. abortus* for its applicability for bovine brucellosis has been documented (Meador and Deyoe 1986; Elzer et al 1996). Typically, studies show the 30-50% of pregnant goats infected with *B. abortus* 2308 abort, and 50-70% of dam/kid pairs are colonized (Elzer et al 2002).

The inoculation, intraveniously or via uterine arteries, of pregnant goats with *B. abortus* revealed placentitis five days post-inoculation and abortions eleven days later (Anderson et al 1986a and 1986b). A study of the histopathology of the bovine pathogen

in pregnant goats in mid-gestation also revealed lesions similar to those of *B. abortus*-infected cows and fetuses/kids although occurring at a less consistent rate in goat fetuses/kids than in bovine fetuses/kids (Meador et al 1998).

Studies have also been done to determine the effects of rough strains of *B. abortus* in goats. A rough strain of *B. abortus*, RB-51, was found to maintain its rough phenotype, produce significant levels of anti-*Brucella* IgG, and did not produce abortions when experimentally injected into the fetuses of goats in their last trimester of pregnancy (Roop et al 1991).

***B. melitensis***

As the primary host of *B. melitensis*, goat infections can be extremely devastating. Caprine brucellosis is characterized by abortion, low production, and infertility among infected animals (Zygmunt et al 2006). The organism enters the animal through the mouth, nose, eye, or an area of abraded skin; and the infection quickly spreads throughout the animal's body via its lymphatic system. In documented studies, pregnant goats infected with *B. melitensis* 16M generally abort the fetus 70-100% of the time with 90-100% of the dam/kid pairs being culture positive (Elzer et al 2002).

Infection of a pregnant uterus almost always ends in abortion of the fetus, which usually occurs during the last two months of pregnancy (Alton 1990). Kids may be born alive and weak or may appear to be healthy. But, upon the culturing of their tissues, the kids are confirmed as being colonized by the organism. Some kids have been found to be culture- and sero-negative by the time they are two months old (Alton 1990). Others have also been observed to be anti-*Brucella* antibody free prior to receiving colostrum (Meador and Deyoe 1986).

## HYPOTHESES

The current study evaluates the virulence of a putative *Brucella melitensis* 16M hemagglutinin gene, Region E, in the caprine model, according to the following hypotheses: production of a *B. abortus* 2308 variant, *B. abortus* 2308-QAE, through the expression of Region E *in trans* causes increased virulence within the goat model and creation of a *Brucella melitensis* 16M mutant, *B. melitensis* 16MΔE, through the disruption of Region E causes attenuation within the goat model. Genetically-manipulated strains of *B. abortus* 2308 and *B. melitensis* 16M were assessed based upon their colonization of non-pregnant goats and dam/kid pairs and the abortion rate of pregnant goats infected with the test strains.

## MATERIALS AND METHODS

### Bacterial Strains

*Brucella abortus* strain 2308 and *B. melitensis* strain 16M as well as *B. abortus* 2308-QAE (this study) and *B. melitensis* 16MΔE (this study) were cultured from the laboratory stock when needed. Glycerol stocks of *B. abortus* 2308 and *B. melitensis* 16M were thawed and plated on Schaedler Brucella Agar (SBA) (Difco Laboratories, Detroit, MI). Glycerol stocks of *B. abortus* 2308-QAE were plated on SBA containing 100 μg/ml ampicillin (Sigma Chemical Company, St. Louis, MO) and *B. melitensis* 16MΔE on SBA containing 45 μg/ml kanamycin (Sigma Chemical Company). Plates were incubated at 37ºC in a 5% $CO_2$ atmosphere for 2-3 days. Bacterial lawns were harvested with sterile brucella broth (Difco Laboratories). Equal volumes of the cell suspensions and a 50% glycerol (EMD Chemicals, Inc., Gibbstown, NJ) - brucella broth mixture were made and stored at -80ºC until needed.

Inoculation doses of *B. abortus* 2308, *B. melitensis* 16M, *B. abortus* 2308-QAE, and *B. melitensis* 16MΔE were made by harvesting 2-3 day-incubated plates with brucella broth. Cell suspensions were diluted and standardized via spectrophotometer (Bausch and Lomb, Rochester, NY) to an O.D. reading of 0.15 at 600λ for a concentration of $1x10^9$ CFU/ml. Infectious doses were snap frozen in liquid nitrogen in 1 ml aliquots and stored at -80ºC until needed. Viability counts on SBA plates, SBA plates with ampicillin (100 μg/ml), and SBA plates with kanamycin (45 μg/ml) using serial dilutions were done to validate the concentration of the inoculation doses.

One Shot® TOP10 Chemically Competent *Escherichia coli* Cells (Invitrogen Corporation, Carlsbad, CA) were obtained commercially. They were received frozen on dry ice and were stored at -80ºC until needed.

**Genomic DNA Preparation**

Genomic DNA was isolated from *B. abortus* 2308 and *B. melitensis* 16M for analysis by Polymerase Chain Reaction (PCR). SBA plates were inoculated with 100 µl of either organism and incubated at 37ºC and in a 5% $CO_2$ atmosphere for 2-3 days. Plates were harvested in 2 ml Phosphate Buffered Saline (PBS) (Sigma Chemical Company). Cell suspensions were placed into 15 ml centrifuge tubes. An equal volume of chloroform ($CH_3Cl$) was added to the cells, and the tubes were rocked in a biosafety cabinet for 1 hour. The mixture was allowed to settle for at least 30 minutes resulting in the formation of distinct layers. The aqueous layer containing the bacteria was removed to another 15 ml centrifuge tube and the remaining $CH_3Cl$ discarded. Two hundred to 500 µl of chloroform-killed cells were transferred to a 1.5 ml micro centrifuge tube and pelleted at 9,000 rpm for 10 minutes and the supernatant discarded. The remaining pelleted cells were used with the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA).

Pelleted cells were re-suspended in 180 µl Buffer ATL (Qiagen, Inc.). Proteinase K (Qiagen, Inc.) was added to the suspension before it was mixed by vortexing and incubated at 55ºC for 1 hour in a shaking water bath. Following incubation, cells were vortexed for 15 seconds and 200 µl Buffer AL (Qiagen, Inc.) was added to the sample before again thoroughly mixed by vortexing. The cells were incubated in a heating block for 10 minutes at 70ºC. Two hundred micro liters absolute ethanol was added to the

sample, and it was mixed by vortexing. The entire mixture was then pipetted into a DNeasy mini column sitting in a 2 ml collection tube (Qiagen, Inc.) and centrifuged at 8,000 rpm for 1 minute. The flow-through and the collection tube were discarded and the mini column placed into another 2 ml collection tube. Five hundred micro liters Buffer AW1 (Qiagen, Inc.) was added to the column. It was centrifuged at 8,000 rpm for 1 minute, and the flow-through and collection tube were discarded. The mini column was again transferred to a new 2 ml collection tube, and 500 µl Buffer AW2 (Qiagen, Inc.) was added to the column and spun at 15,000 rpm for 3 minutes to dry the DNeasy membrane (Qiagen, Inc.). The mini column was placed into a sterile 1.5 ml micro centrifuge tube, and 200 µl Buffer AE (Qiagen, Inc.) was added directly to the membrane. The tube was incubated at room temperature for 1 minute then centrifuged at 8,000 rpm to elute the DNA. The elution step was repeated into the same tube. Isolated DNA was stored at -20ºC until needed.

**Polymerase Chain Reaction**

Polymerase Chain Reaction (PCR) amplification was performed using a MyCycler™ Personal Thermal Cycler (BioRad Laboratories, Inc., Hercules, CA) with the FailSafe™ PCR System with the 2X G PreMix (Epicentre Biotechnologies, Madison, WI). Each reaction contained 0.75 Units FailSafe™ PCR Enzyme Mix (Epicentre Biotechnologies) which consisted of a blend of thermostable DNA polymerases, FailSafe™ PCR 2X G PreMix [100 mM Tris-HCl, 100 mM KCl, 400 µM of each dNTP, and varying concentrations of $MgCl_2$ (3-7 mM) and FailSafe™ PCR Enhancer (0-8X) with betaine], approximately 300 ng of template DNA, and 0.2 µM of each primer

specific for either the *B. melitensis* 16M hemagglutinin gene (Region E) or the kanamycin gene from pBBRIMCS-2 (Kovach et al 1995).

A set of primers was designed for an approximately 2000 bp putative hemagglutinin gene (Region E) in the *B. melitensis* 16M. Primers ORF-944F (5'- GAATTGGCGACCTGACTGAGGA -3') and ORF-944R (5'- CTCACGGCTGTTCTCCTTTAACA -3') were desidned at The Institute of Molecular Biology and Medicine at the University of Scranton (Scranton, PA) from the gene sequence of *B. melitensis* 16M chromosome II (GenBank Accession Number AE008918). Primers were synthesized by BioMMED in the Department of Pathobiological Sciences at the Louisiana State University School of Veterinary Medicine (Baton Rouge, LA). A second set of primers was designed for an 840 bp kanamycin gene from the published nucleotide sequence of pBBR1MCS-2 (GenBank Accession Number U23751). Primers KAN-AL (5'- TGACCGGTTCATTTCGAACCCCAGAGTC -3') and KAN-AR (5'- AGACCGGTACAGGATGAGGATCGTTTCG -3') were constructed with an *Age* I (New England Biolabs, Beverly, MA) endonuclease restriction sequence site (5'- ACCGGT -3') at their 5'- ends for further genetic manipulation. They were initially synthesized by BioMMED in the Department of Pathobiological Sciences at the Louisiana State University School of Veterinary Medicine (Baton Rouge, LA) and subsequently produced by Integrated DNA Technologies, Inc. (Coralville, IA).

For the amplification of Region E, template DNA was initially denatured at 96ºC for 5 minutes. Samples were then denatured at 96ºC for 1 minute, primers annealed at 55ºC for 30 seconds, and primers extended at 72ºC for 2 minutes over a total of 30

cycles. A final extension at 72ºC for 5 minutes was also performed. Amplification of the kanamycin gene from pBBR1MCS was carried out using the same protocol but with an annealing temperature of 56ºC.

**Cell Transformations**

Cell transformations were performed using the TOPO® Cloning and Transformation protocol for One Shot® Chemically Competent Cells (Invitrogen Corporation). Five to ten micro liters of ligation reactions were added to one vial of chemically competent TOP10 *E. coli* cells (Invitrogen Corporation) and mixed gently without pipetting the mixture up and down. The cells and ligation mixtures were incubated on ice for 10-20 minutes then heat-shocked at 42ºC without shaking for 30 seconds in a heating block. The tubes were then immediately transferred to ice, and 250 µl of room temperature S.O.C. medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) (Invitrogen Corporation) was added to each tube. The cells were then incubated at 37ºC for 1 hour in a shaking water bath. Following incubation, 50 µl to 100 µl of each transformation was plated on pre-warmed Luria-Bertani (LB) plates (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.0) with or without ampicillin (100 µg/ml), kanamycin (45 µg/ml), or ampicillin and kanamycin. The plates were incubated at 37º C overnight and observed for growth. Successful transformation colonies were picked with a sterile loop and inoculated into 2 ml to 4 ml LB broth and incubated at 37ºC overnight in a shaking water bath in preparation for plasmid DNA isolation.

**Plasmid Preparation**

Plasmid mini preps were performed on suspect *E. coli* colonies to confirm the presence of plasmids, pQAE and pUC19ΔE, using the Qiagen Buffer System (Qiagen, Inc.). After an overnight incubation at 37ºC in a shaking water bath, approximately 1.5 ml of broth culture was pelleted at 9,000 rpm for 5 minutes and the supernatant discarded. The pellet was completely re-suspended in 300 µl of Buffer P1 (50 mM Tris base; 10 mM EDTA, pH 8.0; 100 µg/ml RNase A) by vortexing. Three hundred micro liters of Buffer P2 (200 mM NaOH, 1% SDS) was added to the tubes and mixed thoroughly before the addition of 300 µl of chilled Buffer P3 (3.0 M $CH_3CO_2K$, pH 5.5). The tubes were mixed by inverting several times then centrifuged at 14,000 rpm for 10 minutes. The supernatant was promptly removed to a clean 1.5 ml micro centrifuge tube and the DNA precipitated with 0.7 times the total volume with isopropanol by inverting the tube several times. Following a 15 minute centrifugation at 14,000 rpm, the supernatant was promptly poured off, and the pellet was allowed to air dry. The pellet was re-suspended in 25-30 µl of $dH_2O$. Glycerol stocks of the re-suspended, confirmed plasmids were made as described above and stored at -20ºC until used. *Brucella spp*. were initially chloroform killed prior to the cells being used in the above mini preps protocol.

Plasmid maxi preps were performed on *E. coli* colonies to acquire a large quantity of isolated pQAE for electroporation into *B. abortus* 2308 using the Qiagen Plasmid Maxi Kit (Qiagen, Inc.). A single colony was picked from a streaked plate into a 2-5 ml LB medium containing 100 µg/ml ampicillin and incubated for ~8 hours at 37ºC in a shaking (300 rpm) water bath. The starter culture was then diluted 1/1000 into 100 ml

LB medium containing 100 µg/ml ampicillin and incubated at 37ºC for 12-16 hours in a shaking (300 rpm) water bath. The cells were harvested by centrifugation at 6,000 rpm for 15 minutes at 4ºC and re-suspended in 10 ml of Buffer P1(50 mM Tris base; 10 mM EDTA, pH 8.0; 100 µg/ml RNase A). Ten milliliters of Buffer P2 (200 mM NaOH, 1% SDS) was added, mixed, and the sample incubated at room temperature for 5 minutes. Following incubation, ten ml of chilled Buffer P3 (3.0 M $CH_3CO_2K$, pH 5.5) was added, mixed gently by inverting, and incubated on ice for 20 minutes. The sample was then centrifuged at ≥20,000 x g for 30 minutes at 4ºC and plasmid-containing supernatant removed and re-centrifuged at ≥20,000 x g for 15 minutes at 4ºC, removing the plasmid-containing supernatant to a Qiagen-tip 500 column, which had been equilibrated with 10 ml Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Trion® X-100). The column was washed two times with 30 ml Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol). The DNA was eluted from the column using 15 ml Buffer QF (1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol), precipitated in 0.7 times the total volume with room temperature isopropanol, and centrifuged at ≥15,000 x g for 30 minutes at 4ºC. Pelleted DNA was washed with 5 ml room temperature 70% ethanol and centrifuged at ≥15,000 x g for 10 minutes. The supernatant was decanted. The pellet was allowed to air dry and was re-suspended in $dH_2O$. Confirmed plasmids were stored at -20ºC until used.

**Agarose Gel Electrophoresis**

DNA products generated via PCR, endonuclease restriction digestions, genomic DNA isolations, or plasmid DNA preparations were mixed with 3 µl to 5 µl 6X loading buffer (12% Ficoll 4000, 1.0 M $Na_2EDTA$, pH 8, 0.6% SDS, 0.15% bromphenol blue)

and visualized by agarose gel electrophoresis. DNA products were run on 0.7% to 1% ultra pure agarose (Invitrogen Corporation)-TAE (Tris-base, glacial acetic acid, $Na_2EDTA$) gel. Gels were run at 100 volts in a BioRad DNA Sub Cell (BioRad Laboratories, Inc.) until the dye front was approximately 1 inch from the bottom of the gel. One hundred base pair or 1 kb molecular weight standards (New England Biolabs,) were visualized on gels to determine the size and approximate concentration of linearized DNA samples being tested. One Kilobase Supercoiled Plasmid Ladder (Bayou Biolabs, Harahan, LA) was visualized on gels to determine the size and approximate concentration of supercoiled plasmid DNA samples. Gels were incubated in 1.0 µg/ml ethidium bromide for 5-8 minutes and destained in $dH_2O$ for 30 minutes. DNA bands within the gels were viewed and photographed using a Polaroid Gel Documentation System (BioRad Laboratories, Inc.) which included a UV Transilluminator.

**Gel Purification**

DNA products derived from PCR, endonuclease restriction digestions, genomic DNA isolations, or plasmid DNA preparations were routinely purified via agarose gel purification before they were used in any additional reactions. Gel purification was achieved using a Zymoclean Gel DNA Recovery Kit™ (Zymo Research, Orange, CA). Bands of interest were excised from the agarose gels with a sterile razor blade or scalpel and transferred to a 1.5 ml micro centrifuge tube. Three volumes of ADB-Buffer (Zymo Research) was added to each volume of agarose gel section per tube and incubated at 55ºC in a heating block for 10 minutes until the gel was completely dissolved. The melted gel solution was loaded into a Zymo-spin column and collection tube (Zymo Research) and centrifuged at full speed (15,000 rpm) for 10 seconds. After discarding the

flow-through, 200 µl of Wash Buffer was added to the column, and it was spun at full speed (up to 15,000 rpm) for 10 seconds. The column was then washed again with 200 µl of Wash Buffer and spun at full speed (up to 15,000 rpm) for 30 seconds. Afterwards, the column was transferred to a clean 1.5 ml micro centrifuge tube, and 6 µl to 8 µl sterile $dH_2O$ was added to the column matrix. The tube was spun briefly at full speed (up to 15,000 rpm) to elute the DNA which was stored at -20ºC until used.

**Construction of Plasmids**

A 4,950 bp plasmid, pBBR1MCS-4 (Kovach et al 1995) was digested in a reaction using 20 units of the restriction endonuclease *Eco*R V (New England Biolabs) along with 2.5 µl 1X NEBuffer 3, 2.5 µl 100 µg/ml bovine serum albumin (BSA), and $dH_2O$ to a volume of 25 µl and incubated at 37ºC for 1 hour with gentle agitation in a shaking water bath. The enzyme was deactivated at 85ºC for up to 20 minutes in a heating block. The digested samples were run on 0.7-1.0% agarose gels for visualization of linearized plasmids and then gel purified as described above. PCR-amplified Region E was ligated into the *Eco*R V-linearized, gel-purified pBBR1MCS-4 plasmid using the Fast-Link™ DNA Ligation Kit for Blunt End Ligation (Epicentre Biotechnologies) to create pQAE. Region E and the *Eco*R V-digested pBBR1MCS-4 were combined in a 15 µl total volume reaction with 1.5 µl 10X Last-Link Ligation Buffer, 0.75 µl 10mM ATP, a 1:5 concentration ratio of vector DNA to insert DNA, sterile $dH_2O$ to 14 µl, and 1 µl Fast-Link DNA Ligase. The reaction was incubated at room temperature for 15 minutes and then transferred to a 70ºC heating block for 15 minutes to inactivate the ligase. The reaction was briefly spun at full speed (15,000 rpm) in a micro centrifuge tube. The ligation mixture was then used to transform One Shot® Chemically Competent Cells as

described above and plated on LB agar plates supplemented with 100 µg/ml ampicillin and incubated overnight at 37ºC. Successful transformants were cultured and their plasmids isolated as described above for confirmation of the new 6,950 bp plasmid, pQAE. Confirmation was achieved via PCR amplification using the Region E primers and enzyme digestion with *Eco*R V. All techniques were preformed as described above. Maxi preps were done on cultured One Shot® Chemically Competent Cells containing pQAE as described above, and the isolated plasmid DNA was electroporated into *B. abortus* 2308 or frozen at -20ºC until further use.

A ~2.0 kilobase PCR-amplified Region E fragment was generated from *B. melitensis* 16M genomic DNA using the primers ORF-944F and ORF-944R as described above. The PCR fragment was then ligated into the linear 3,519 base pair pCR®-Blunt II-TOPO® vector (Invitrogen Corporation) using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen Corporation). Three micro liters of fresh PCR product, 1 µl of kit-supplied salt solution, 1 µl sterile $dH_2O$ and 1 µl TOPO® vector were added for a final volume of 6 µl in a micro centrifuge tube. The reaction was gently mixed by fingertip-tapping and incubated at room temperature for 20 minutes. The cloning reaction was then used in the TOPO® Cloning and Transformation protocol with One Shot® Chemically Competent Cells (Invitrogen Corporation) as described above and plated on LB agar plates supplemented with 45 g/ml kanamycin and incubated overnight at 37ºC. Successful transformants were cultured and their plasmids isolated for confirmation of pTOPO+E as described above.

Region E was then excised from the TOPO® vector via *Eco*R I (New England Biolabs) endonuclease restriction digestion. A 2,686 *E. coli* plasmid vector, pUC19

(Yanisch-Perron et al 1985), which confers ampicillin resistance, was also digested using *Eco*R I. In separate 25 µl reactions, pTOPO+E and pUC19 were each combined with 2.5 µl 1X NEBuffer for *Eco*R I, 20 units *Eco*R I, and $dH_2O$ to a total volume of 25 µl. The reactions were incubated at 37ºC for 1 hour with gentle agitation in a shaking water bath to release the Region E fragment and the linearized pUC19 vector, leaving both with *Eco*R I overhanging 3' and 5' ends. The enzyme was then deactivated in a 65ºC heating block for up to 20 minutes. The digested samples were run on 0.7-1.0% agarose gels for visualization of the released Region E and the linearized pUC19 and then gel purified as described above.

The *Eco*R I-digested Region E and pUC19 were ligated together using the Fast-Link™ DNA Ligation Kit for Cohesive End Ligation (Epicentre Biotechnologies) to create pUC19E. Region E and pUC19 were combined in a 15 µl total volume reaction with 1.5 µl 10X Last-Link Ligation Buffer, 1.5 µl 10mM ATP, a 1:2 concentration ratio of vector DNA to insert DNA, sterile $dH_2O$ to 14 µl, and 1 µl Fast-Link DNA Ligase. The reaction was incubated at room temperature for 15 minutes and then transferred to a 70ºC heating block for 5 minutes to inactivate the ligase. The reaction was briefly spun at full speed (up to 15,000 rpm) in a micro centrifuge tube. The ligation mixture was then used to transform One Shot® Chemically Competent Cells as described above and plated on LB agar plates supplemented with 100 µg/ml ampicillin and incubated overnight at 37ºC. Successful transformants were cultured and their plasmids isolated as described above for confirmation of pUC19E, a 4,686 bp vector.

Disruption of Region E on the pUC19E plasmid was achieved by excising a 1.2 bp interior portion of Region E. An 840 bp kanamycin cassette was PCR-amplified from the broad-host-range vector, pBBR1MCS-2 (Kovach et al 1995) using the primers KAN-AL and KAN-AR, containing the *Age* I restriction endonuclease sequence, 5'-ACCGGT-3', added to their 5' ends as described above. Both pUC19E and the PCR-amplified kanamycin antibiotic cassette (KAN) were digested with *Age* I in a 25 µl reaction containing 2.5 µl 1X NEBuffer 1, 5 units of *Age* I, and $dH_20$ to a total volume of 25 µl. The reactions were incubated at 37ºC for 1 hour with gentle agitation in a shaking water bath, which linearized pUC19E, releasing the 1.2 bp internal portion of Region E and digested KAN cassette leaving both with *Age* I overhanging 3' and 5' ends. The enzyme was then deactivated in a 65ºC heating block for up to 20 minutes. The digested samples were run on 0.7-1.0% agarose gels for visualization of the released interior portion of Region E and the linearized pUC19E and then gel purified as described above.

*Age* I-digested pUC19E and kanamycin cassette were ligated together using the Fast-Link™ DNA Ligation Kit for Cohesive End Ligation (Epicentre Biotechnologies) to create pUC19ΔE. The kanamycin cassette and pUC19E were combined in a 15 µl total volume reaction with 1.5 µl 10X Last-Link Ligation Buffer, 1.5 µl 10mM ATP, a 1:2 concentration ratio of vector DNA to insert DNA, sterile $dH_2O$ to 14 µl, and 1 µl Fast-Link DNA Ligase. The reaction was incubated at room temperature for 15 minutes and then transferred to a 70ºC heating block for 5 minutes to inactivate the ligase. The reaction was briefly spun at full speed (up to 15,000 rpm) in a micro centrifuge tube. The ligation mixture was then used to transform One Shot® Chemically Competent Cells as described above and plated on LB agar plates supplemented with 45 µg/ml kanamycin

and 100 µg/ml ampicillin and incubated overnight at 37ºC. Successful transformants were cultured and their plasmids isolated as described above for confirmation of pUC19ΔE, a 4,326 bp vector.

The new plasmid, pUC19ΔE, was digested with *Eco*R I to produce the 1,640 Region E-Kanamycin-Region E (E-KAN-E) fragment. The reaction contained the plasmid, pUC19ΔE, along with 2.5 µl 1X NEBuffer for *Eco*R I, 20 Units *Eco*R I, and $dH_2O$ to volume. The reaction was incubated at 37ºC for 1 hour with gentle agitation in a shaking water bath to release the E-KAN-E fragment. The enzyme was then deactivated in a 65ºC heating block for up to 20 minutes. The digested sample was run on 0.7-1.0% agarose gels for visualization of the released E-KAN-E fragment and gel purified as described above. The fragment was stored at -20ºC until it was electroporated into *B. melitensis* 16M.

**Electroporation**

A 100 µl infectious dose containing $1x10^9$ CFU/ml of *B. abortus* 2308 or *B. melitensis* 16M was thawed and plated on Schaedler Agar (Difco Laboratories) supplemented with 5% whole bovine blood taken from an animal with no evidence of *Brucella* exposure. Following a 2-3 day incubation period at 37°C in a 5% $CO_2$ atmosphere, the bacterial cells were harvested in 2 ml of sterile $dH_2O$. Twenty-five milliliters of sterile Brucella broth (Difco Laboratories) was inoculated with the harvested cells and incubated overnight at 37°C in a shaking water bath. After incubation, the culture was equally dispensed into micro centrifuge tubes and spun at 6,000 rpm for 15 minutes. Combined pellets were re-suspended in a total volume of 1 ml cold sterile $dH_2O$ and centrifuged at 15,000 rpm for 5 minutes and the supernatant discarded. The resulting

pellet was then washed three additional times with 1 ml cold sterile $dH_2O$ with the final pellet re-suspended in 100 µl of cold sterile $dH_2O$.

Electroporations were performed using an Eppendorf 2510 Electroporator (Eppendorf Scientific, Inc., Madison, WI). Thirty-three micro liters of a cold *B. abortus* 2308 cell suspension with 3 µl of a cold pQAE plasmid solution or 33 µl of a cold *B. melitensis* 16M cell suspension with 4 µl of a cold E-Kan-E fragment solution were combined in a pre-chilled 2 mm, 0.04 ml Eppendorf Electroporation Cuvette (Brinkmann Instruments, Inc., Westbury, NY). The mixtures were electroporated at 2.5 kV for approximately 5-6 milliseconds. Five hundred micro liters of cold filter-sterilized SOC-B (6% trypticase soy broth (Difco), 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) (Lai et al 1990) recovery media was immediately added to the cells which were incubated overnight at 37°C in a shaking water bath.

One hundred micro liters of the electroporated cells were plated onto SBA plates containing either 100 µg/ml ampicillin (Sigma Chemical Company) for the *B. abortus* 2308-QAE cells or 45 µg/ml kanamycin (Sigma Chemical Company) for the *B. melitensis* 16MΔE cells. Plates were incubated at 37ºC in a 5% $CO_2$ atmosphere and observed for growth for 2 weeks. All resultant *B. abortus* 2308-QAE colonies were patched onto SBA plates containing ampicillin (100 µg/ml), and all resultant *B. melitensis* 16MΔE colonies were patched onto SBA plates containing kanamycin (45 µg/ml). Plates were incubated at 37ºC at a 5% $CO_2$ atmosphere and observed for growth.

**Standard *Brucella* Identification Tests**

Potential mutant colonies were isolated for *Brucella* speciation using techniques commonly performed to differentiate *Brucella spp*. from other Gram-negative organisms.

An organism's ability to break down urea into ammonia, $H_2O$, and $CO_2$ because of its production of urease was evaluated using urease slants (Alton et al 1988). An isolated colony was streaked onto a urease slant using a sterile loop and incubated at 37ºC in a 5% $CO_2$ atmosphere for 24-48 hours. A pH indicator in the media turned the yellow media pink in the presence of the alkaline ammonia product to indicate a positive reaction.

The presence of a cytochrome oxidase, which aids in reducing molecular oxygen by an organism, was evaluated using the oxidase test. A commercial oxidase reagent ampule (Becton Dickinson and Company, Cockeysville, MD) was broken, and its contents mixed. Colonies were picked using a sterile cotton swabs. A few drops of the oxidase reagent were added to the colonies on the swabs. Within 30 seconds, a violet to purple color appeared on the picked colonies if the organisms were oxidase positive.

The catalase test was performed to examine an organism's ability to break down hydrogen peroxide into water and oxygen. A commercially-available catalase reagent dropper (Becton Dickinson and Company) was used. Suspected colonies were picked using a sterile loop and smeared onto a clean glass slide. One to two drops of the reagent were added to the smear and mixed. The immediate appearance of gas bubbles was indicative of a positive catalase reaction.

**Goats**

For the colonization studies, 24 adult male and female Spanish goats were obtained from commercial herds or from the Elzer farm herd (Louisiana State University, Baton Rouge, LA). All animal sera samples were Brucellosis Card tested and evaluated by western blot prior to any experimentation to confirm the absence of Brucella-specific antibodies. The animals were housed throughout the study at the Ben Hur Large Animal

Isolation Facility, a restricted-access USDA/APHIS/VS/CDC –approved facility. All animals were cared for in accordance with the LSU AgCenter Animal Care and Use Committee guidelines.

For the pathogenesis studies, 37 pregnant Angora and/or Spanish goats were obtained from commercial herds or from the Elzer farm herd. Eight sexually-mature, female Spanish goats obtained from commercial herds or from the Elzer farm herd were administered two doses of Lutalyse® (dinoprost tromethamine) Sterile Solution (Upjohn Co., Kalamazoo, MI) intramuscularly with an 11 day interval between doses for estrus synchronization. The first dose was 15 mg in 3 ml, and the second was ~8 mg in 1.6 ml. Dams were bred with *Brucella*-negative billies, and their pregnancies were later confirmed via ultrasound. All animal sera samples were Brucellosis Card tested and evaluated by western blot prior to any experimentation to confirm the absence of Brucella-specific antibodies. The animals were housed throughout the study at the Ben Hur Large Animal Isolation Facility, a restricted-access USDA/APHIS/VS/CDC – approved facility. All animals were cared for in accordance with the LSU AgCenter Animal Care and Use Committee guidelines.

**Serum Collection**

Goats were bled using 20 gauge, 1.0 inch Precision Glide® Vacutainer® Brand Blood Collection needles (Becton Dickinson and Company, Franklin Lakes, NJ) along with sterile 10 ml BD Vacutainer® Serum tubes (Becton Dickinson and Company). After separation by centrifugation, sera were stored at -20ºC until tested.

**Serological Analysis**

Pre- and post-inoculation serum samples were analyzed by the Brucellosis Card Test (Becton Dickinson and Company) and western immunoblot. Serum samples were examined via Card Test by placing 30 µl of test serum along with 30 µl stained Buffered Brucella Antigen (BBA) (Becton Dickinson and Company) onto the test area of the Brewer Diagnostic Card (Becton Dickinson and Company). Serum samples and the antigen were mixed to a uniform suspension, rocked back and forth on the card for 4 minutes and then observed for antigen-antibody agglutination.

For western immunoblot analysis, cell lysates of *B. abortus* 2308, *B. melitensis* 16M, *B. abortus* 2308-QAE, and *B. melitensis* 16MΔE were prepared by plating 100 µl glycerol stock of each onto SBA plates with or without the appropriate antibiotic. Cells were cultured for 2-3 days at 37ºC in a 5% $CO_2$ atmosphere then harvested from each plate in 2 ml sterile PBS (Sigma Chemical Company). The resulting suspensions were sonicated for 8 minutes using a Heat Systems-Ultrasonics W-385 Sonicator (Farmingdale, NY) with a tapered micro tip using a 1 second pulse at 50% duty and an output of approximately 4.0 to 4.5. Cells were boiled for 10 minutes to ensure killing of *Brucella*. Lysates were then boiled for 10 minutes in a 1:1 dilution with Laemmli Sample Buffer (BioRad Laboratories). Cell lysates were stored at -20ºC until used.

Cell lysates ranging in volumes of 2 µl to 10 µl were separated by polyacrylamide gel electrophoresis (SDS-PAGE) using 12% Tris-HCl Ready Gels (BioRad Laboratories) run at 150 volts for 1 hour and 18 minutes in a 2-gel Criterion cell as a part of the Criterion Precast Gel System (BioRad Laboratories) . Two micro liters of BioRad Kaleidoscope Prestained Standard (BioRad Laboratories) per blot was used as a

molecular weight marker. At the completion of a run, gel proteins were transferred to a nitrocellulose membrane (Osmotics, Livermore, CA) using a Criterion blotter (BioRad Laboratories) at 100 volts for 1 hour. Following transfer, blots were blocked with 5% Blotting Grade Blocker Nonfat Milk (BioRad Laboratories) for 1 hour on a shaker at room temperature. Blots were then washed 5 times with Tris-Buffered Saline (TBS)-Tween and once with TBS (0.5 M NaCl, 20 mM Tris). Individual blots were incubated in a 1:40 dilution of test serum on a shaker at room temperature overnight. After incubation, blots were again washed with TBS-Tween and TBS and incubated on a shaker for 45 minutes at room temperature in a 1:800 dilution of rabbit anti-goat IgG peroxidase conjugate (Sigma-Aldrich Co., St. Louis, MO). Blots were developed using 4-chloro-1 napthol tablets (Sigma-Aldrich Co.) in a TBS-methanol-3% hydrogen peroxide solution. Reactions were stopped by the addition of $dH_2O$, and blots were allowed to air dry.

**Necropsy**

In the colonization studies, adult non-pregnant goats were euthanized by captive-bolt and exsanguination. Blood samples were collected and tested serologically as described above. The following tissues were collected: parotid, prescapular, internal iliac, supramammary (females), inguinal (males) lymph nodes; liver; and spleen.

In the pathogenesis studies, pregnancies were monitored until delivery and kids recorded as aborted/weak or live/healthy. Live kids were euthanized by $CO_2$ asphyxiation, and lung and abomasal fluid was collected on all kids born or aborted. A month following the last birth or abortion, all dams were euthanized by captive-bolt and exsanguination. Blood samples were collected and tested serologically as described

above. The following tissues were collected: parotid, prescapular, internal iliac, supramammary, lymph nodes; liver; spleen; and mammary gland. All tissues collected were stored at -20ºC until bacteriological analysis.

**Bacteriological Analysis**

Tissue samples were thawed, weighed, and homogenized in 20 ml PBS. One hundred microliters of each sample, including 100 µl abomasal fluid, was plated on SBA plates supplemented with *Brucella* Selective Supplement (Oxoid Ltd., Basingstoke, Hampshire, England) (Farrell 1974). After a 14-day incubation period at 37ºC in a 5% $CO_2$ atmosphere, the total number of colonies present on each plate was counted and CFU/gram of tissue calculated. *Brucella spp*. were identified by colony morphology; growth rate; and urease, oxidase, and catalase reactions. *B. abortus* 2308-QAE was differentiated from *B. abortus* 2308 based upon its ability to grow on SBA plates containing 100 µg/ml ampicillin. *B. melitensis* 16MΔE was differentiated from *B. melitensis* 16M by its ability to grow on SBA plates containing 45 µg/ml kanamycin.

**Statistics**

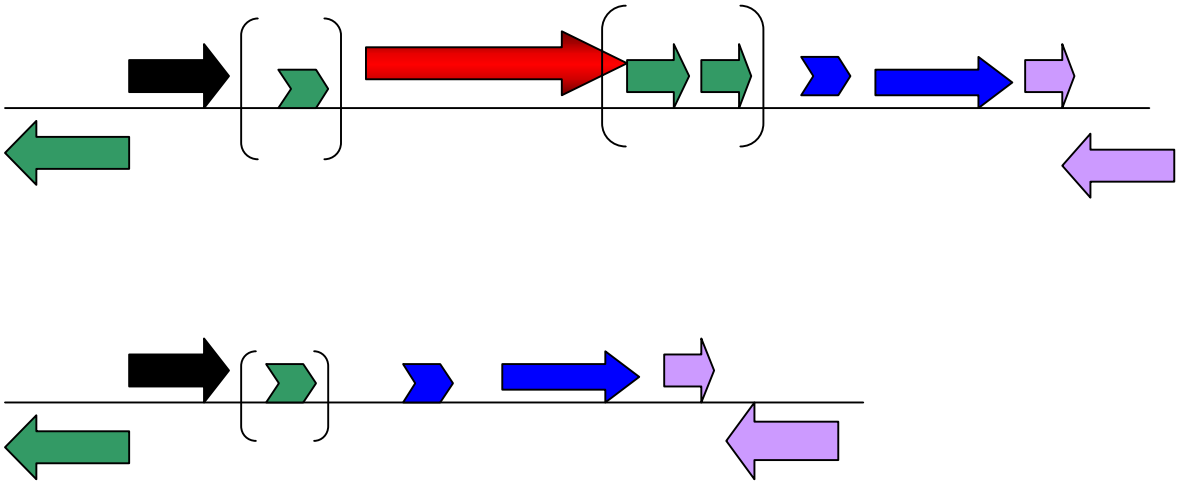
Numbers of colonized dams, colonized kids, and abortions in the pathogenesis study were compared between two groups at a time using a Fisher exact probability test, with $p<0.05$ being considered significant (Snedecor and Cochran 1989). Statistical analysis was performed with statistical software (Sigma Stat Statistical Software, Version 1.0; Jandel Scientific, San Rafael, CA, USA).

## RESULTS

### Creation and Transformation of pQAE

A putative hemagglutinin gene, Region E, was found within the *B. melitensis* genome that is absent in *B. abortus* (Del Vecchio et al 2002b) (Figure 1). A NCBI Nucleotide Blast of the sequence reveals that the gene corresponds to a *B. melitensis* 16M hemagglutinin (GenBank GI 17989062). There is also some homology to a cell wall surface protein of *B. suis* 1330 (GenBank GI 23500299) (Paulsen et al 2002).

***B. melitensis* 16M**



***B. abortus* 2308**

**Figure 1.** Schematic diagram showing the presence and absence of a putative hemagglutinin ORF in *B. melitensis* 16M and *B. abortus* 2308. The hemagglutinin ORF is indicated by the red arrow. Other ORFs in the diagram encode the following proteins: green, transposases; black, preprotein translocase subunit; blue, transcriptional regulator proteins; and purple, hypothetical proteins. [ ] indicates IS elements.

This putative *B. melitensis* hemagglutinin gene was PCR-amplified from the genomic DNA of *B. melitensis* 16M using the primers ORF-944F and ORF-944R (Figure 2). The primers are specific for the ~2.0 kilobase hemagglutinin gene of *B. melitensis* 16M, which is not present in the *B. abortus* 2308 genome. The PCR fragment was visualized by gel electrophoresis (Figure 3) and then gel-purified for further genetic manipulation. Region E, the purified PCR product, was cloned into the 4,950 base pair broad-host-range vector pBBR1MCS-4 (Kovach et al 1995) (Figure 4) which confers ampicillin resistance. Following blunt-end ligation, chemically-competent TOP10 *E. coli* cells (One Shot®) were transformed with the 6,950 base pair plasmid pQAE and plated on LB agar plates supplemented with 100 µg/ml ampicillin and monitored for growth. Minipreps were performed on successful transformants (Figure 5). Resulting plasmids were digested with *Eco*R V to confirm the presence of pQAE by the release of a ~2.0 kilobase Region E fragment from a linear 4.9 kilobase pBBR1MCS-4 plasmid (Figure 6). PCR amplification using ORF-944F/R primers was also performed for additional confirmation of the new plasmid (Figure 7).

**Creation of the *B. abortus* 2308-QAE Variant**

Expression of pQAE *in trans* in *B. abortus* 2308 was achieved by the introduction and maintenance of the low copy number plasmid into the cell (Figure 4). The new plasmid containing Region E from *B. melitensis*, pQAE, was electroporated into *B. abortus* 2308 and screened for successful transformation using SBA plates supplemented with 100 µg/ml ampicillin. Plasmid minipreps were performed on colonies displaying ampicillin resistance, and the presence of pQAE was visualized by gel electrophoresis. Resulting plasmids were also digested with *Eco*R V to confirm the presence of pQAE by

**Region E**

GAATTGGCGACCTGACTGAGGATAGCGGCACAAATACGGCTGCAGTCAGCGGCAATACCATTCGCGCCCAG
ACGATCGTGAATGATAGTTCTAATACCCTTGAAGGAAAACTGTCGGACGACTATGCGTCATCAACGTTGGG
TACCTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGCAATCCTGG
CAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGAGGGCATAATAGGAACCCAG
AATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAGTCTGCAAGAAAATACTATTGATGCTTCGCT
TGCCGTCAACAATGCGGCGAACAGGATTGCCATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCC
TTATTACCAATCTGCAGCAGAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCG
CAAGCGACAGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAACAT
TGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCATCAGATTGTGATCG
GTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTCAACGATATCGCATGACGGAGGCTCT
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TCATTTGACAATCTCCAGCGCGTCTATTGGTGTGGAGGGTACACCGACGATTGGTGCGGTTGTTGATGCCG
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CGTTAAACAGGAGGAATCCAGCTTTTCGGTAACCGACAATGTGCAGAGCGCCTCAGCCAGCGGCACCCAGA
GCCGCAATGAACTGGTGGTCGAGAGCAATTCCGTCACTGCCAATATTGGAACTGGTGCTCAAGAGCATCCC
ACTTCATCAAATACCGGTCTTGACGGAGCGTATGTACGCGATAACGAAGATAGTTTCCATACCATTCATCA
GCCTATGATCATGGCGGCCTATGGCCTTATCAACGATCAGTCGATTGGGGGGAGAGTCAATGCCCTAGAAC
CTGTCTGCATTCAAGGATTCCCTTTTGTACGAAATTCTGATTCAAGGTTGTTAAAGGAGAACAGCCGTGAG

**Figure 2.** Region E isolated within the ORF-944F and ORF-944R primers.

Primers ORF-944F (5'- GAATTGGCGACCTGACTGAGGA -3') and ORF-944R (5'- CTCACGGCTGTTCTCCTTTAACA -3') are shown in red.

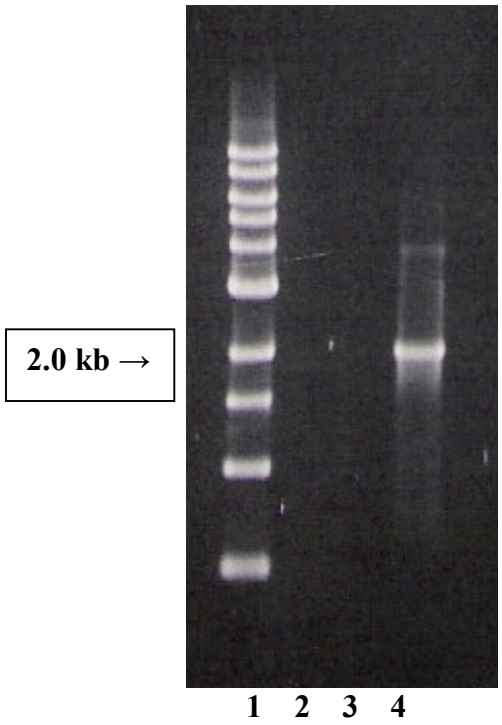


**Figure 3.** Agarose gel electrophoresis of the ~2.0 kilobase Region E PCR-amplified using the ORF-944F and ORF-944R primers. The arrow indicates the 2.0 kb band on the molecular weight ladder.

Lane 1 - 1 kb Molecular Weight Ladder
Lane 2 - $dH_2O$ negative control
Lane 3 - *B. abortus* 2308 Genomic DNA
Lane 4 - *B. melitensis* 16M Genomic DNA

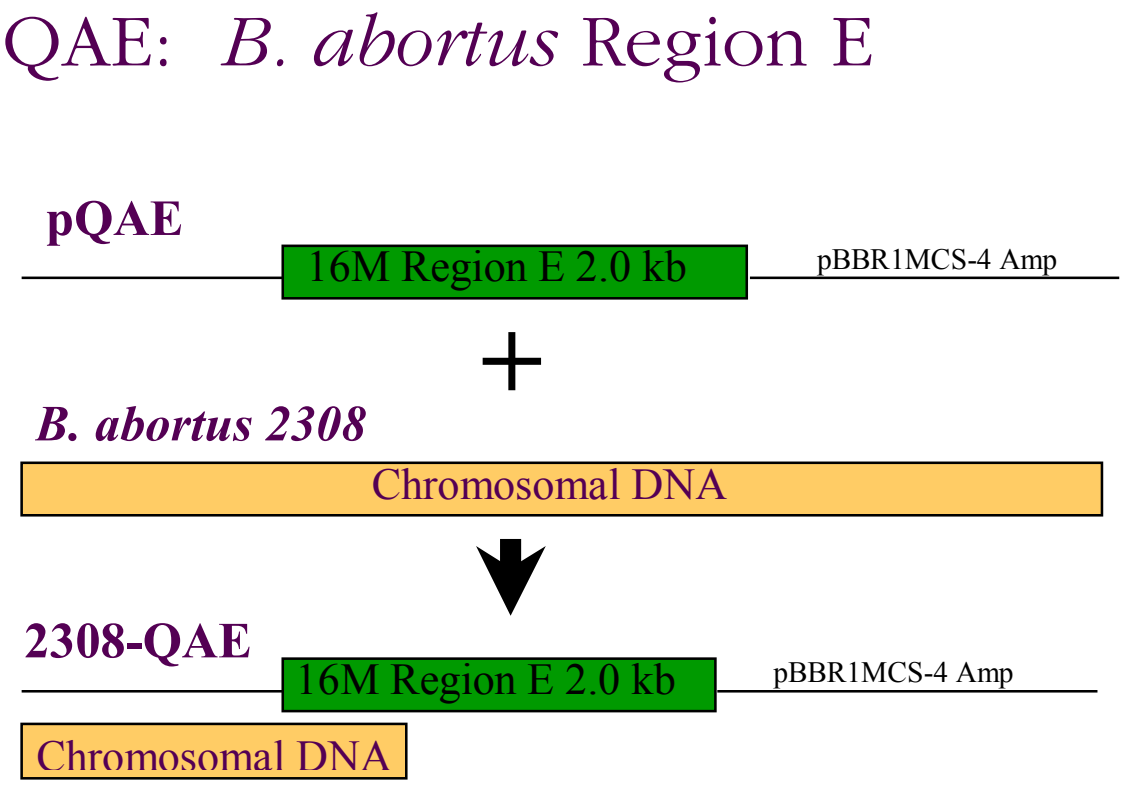


**Figure 4**. Schematic of the creation of *B. abortus* 2308-QAE.

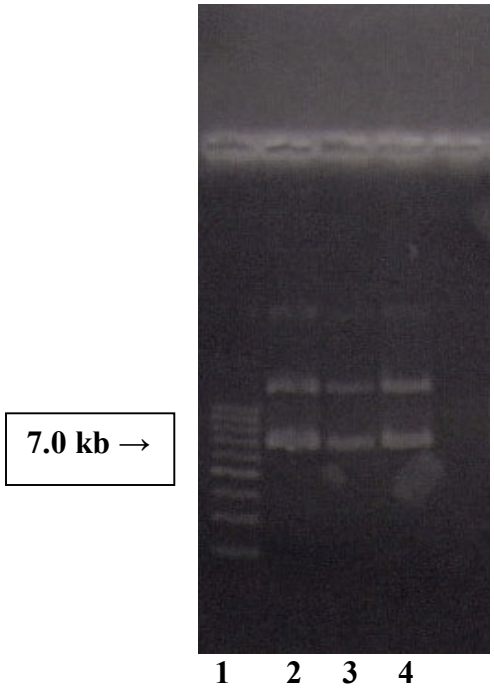


**Figure 5.** Agarose gel electrophoresis of pQAE following plasmid miniprep. The arrow indicates a 7.0 kb band on the molecular weight ladder.

Lane 1 - Supercoiled DNA Molecular Weight Ladder
Lanes 2-4 - pQAE DNA (~6,950 bp)

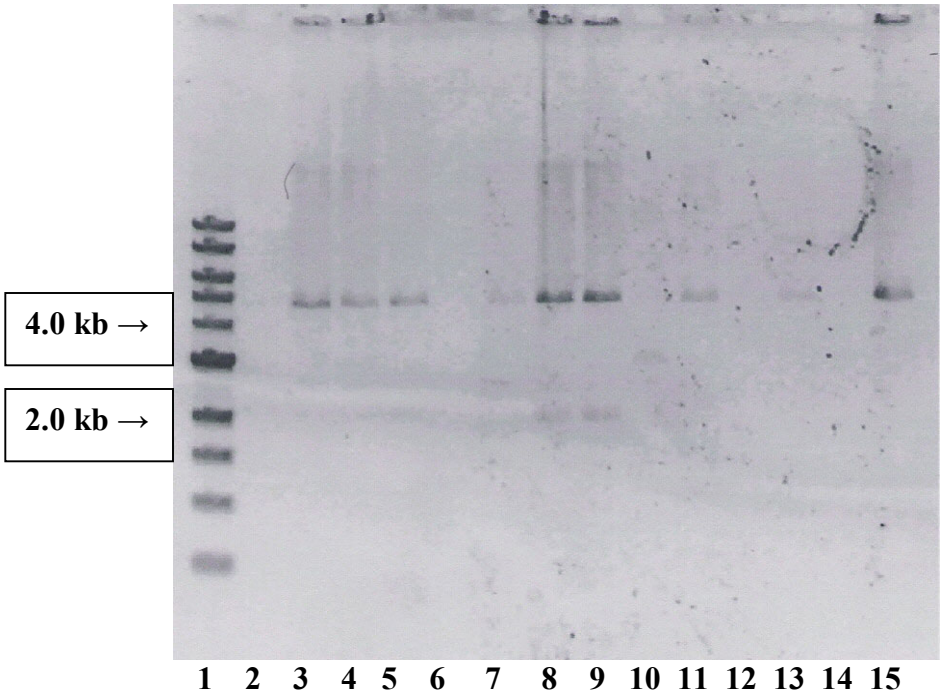


**Figure 6.** Agarose gel electrophoresis of pQAE miniprep DNA digested with *Eco*R V. Digestion reveals a ~2.0 kb Region E and a 4,950 bp linearized pBBR1MCS-4. The arrows indicate 2.0 and 4.0 kb bands on the molecular weight ladder.

Lane 1 - 1 kb Molecular Weight Ladder
Lanes 2-15 - pQAE DNA digested with *Eco*R V

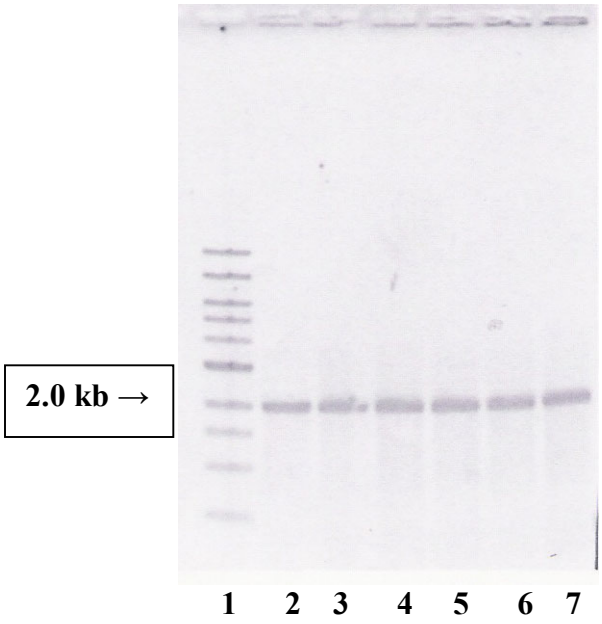


**Figure 7.** Agarose gel electrophoresis of PCR products amplified from pQAE miniprep DNA using the ORF-944F and ORF-944R primers. Gel reveals a ~2.0 kb Region E. The arrow indicates a 2.0 kb band on the molecular weight marker.

Lane 1 - 1 kb Molecular Weight Ladder
Lanes 2-7 - Region E PCR products from pQAE DNA

the release of a ~2.0 kilobase Region E fragment from a linearized 4.9 kilobase pBBR1MCS-4 plasmid (data not shown). The new variant of *B. abortus* 2308 was named *B. abortus* 2308-QAE.

**Creation and Transformation of pUC19ΔE**

Region E, the ~2.0 kilobase PCR fragment, amplified from *B. melitensis* 16M genomic DNA using the primers ORF-944F and ORF-944R (Figure 2) was cloned into the 3,519 base pair pCR®-Blunt II-TOPO® vector (Invitrogen Corporation, Carlsbad, CA) and then excised from the vector via *Eco*R I endonuclease restriction digestion (Figure 8). A 2,686 *E. coli* plasmid vector pUC19 (Yanisch-Perron et al 1985), which confers ampicillin resistance, was also digested using *Eco*R I. These *Eco*R I digestions produced overhanging, cohesive ends on both the PCR product of Region E and the pUC19 vector for uncomplicated ligation of the fragment into the vector producing, a 4,686 base pair plasmid pUC19E.

An 840 base pair kanamycin cassette was amplified from the broad-host-range vector pBBR1MCS-2 (Kovach et al 1995) using the primers KAN-AL and KAN-AR, which contained the *Age* I restriction endonuclease sequence 5'-ACCGGT-3' added to their 5' ends (Figure 9). Both pUC19E and the PCR-amplified kanamycin antibiotic cassette were digested with *Age* I. *Age* I digestion of pUC19E released a 1.2 kilobase internal portion of Region E, which was replaced via cohesive-end ligation with the 840 base pair kanamycin cassette to produce the 4,326 base pair pUC19ΔE (Figure 10). Each PCR amplification and digestion step was visualized by gel electrophoresis, and all DNA components were gel-purified prior to further manipulation. The ~4.3 kilobase pUC19ΔE, which confers ampicillin and kanamycin resistance, was transformed into

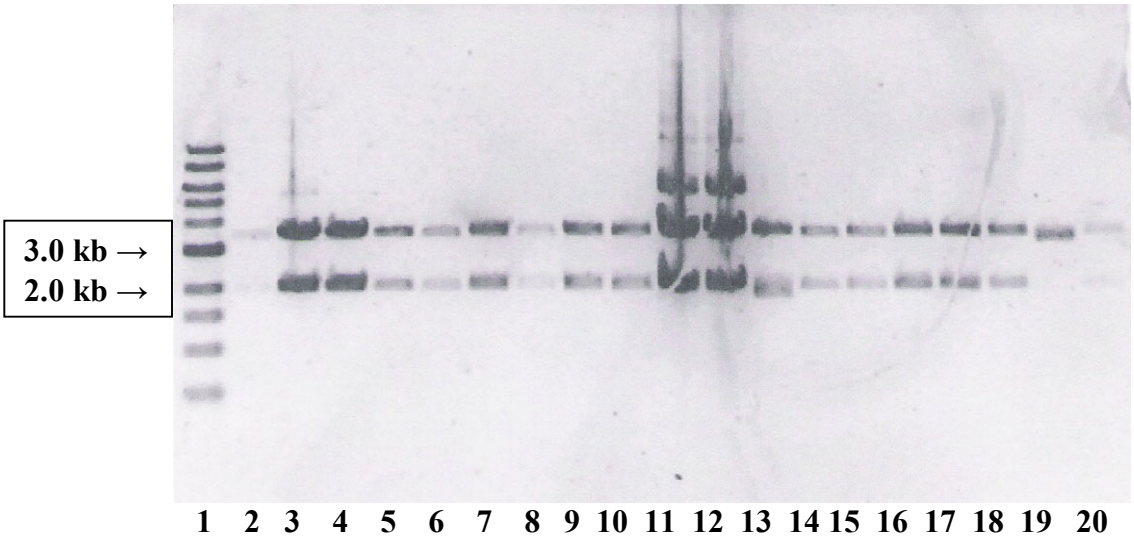


**Figure 8.** Agarose gel electrophoresis of the pCR®-Blunt II-TOPO® vector+Region E miniprep DNA digested with *Eco*R I. Digestion reveals a ~2.0 kb Region E fragment and a 3,519 bp linearized pCR®-Blunt II-TOPO® vector. The arrows indicate 2.0 and 3.0 kb bands on the molecular weight ladder.

Lane 1 - 1 kb Molecular Weight Ladder
Lanes 2-20 - pCR®-Blunt II-TOPO® vector+Region E miniprep DNA digested with *Eco*R I

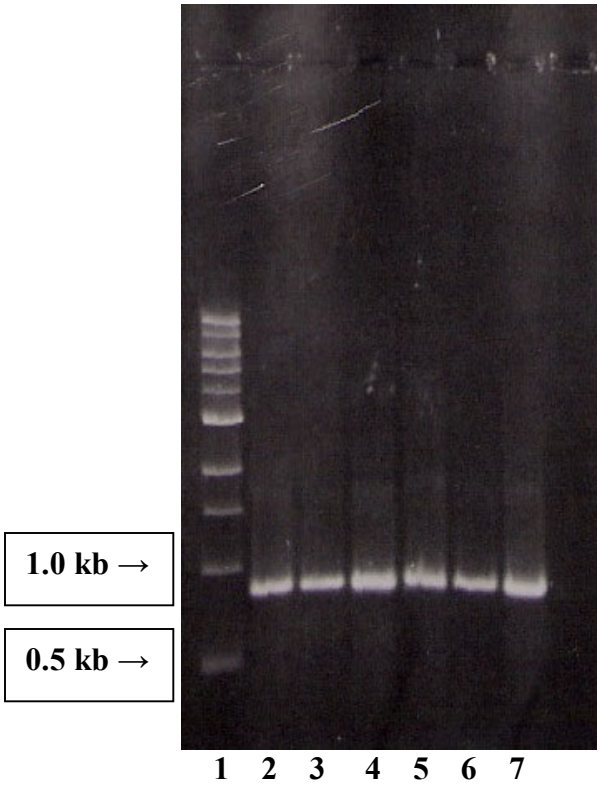


**Figure 9.** Agarose gel electrophoresis of an 840 bp kanamycin antibiotic cassette PCR-amplified from pBBR1MCS-2 using the KAN-AL and KAN-AR primers. The arrows indicate 0.5 and 1.0 kb bands on the molecular weight ladder.

Lane 1 - 100 bp Molecular Weight Ladder
Lanes 2-7 - PCR-amplified kanamycin antibiotic cassette

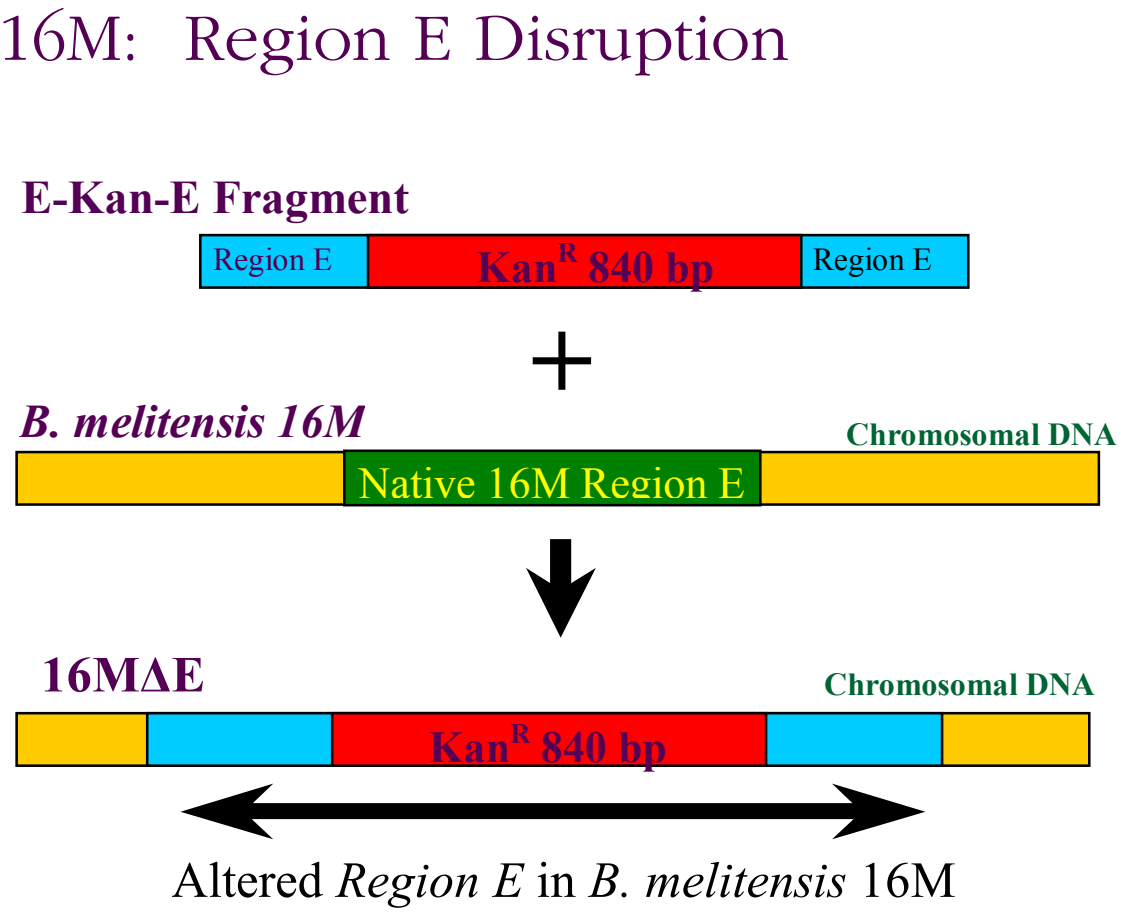


**Figure 10.** Schematic of the creation of *B. melitensis* 16MΔE.

chemically-competent TOP10 *E. coli* cells (One Shot®) and plated on LB agar plates supplemented with 100 µg/ml ampicillin and 45 µg/ml kanamycin. Minipreps were preformed on successful transformants (Figure 11), and the resulting plasmids were digested with *Age* I (Figure 12) and *Eco*R I (data not shown) to confirm the presence of pUC19ΔE. The presence pUC19ΔE was also confirmed using the ORF-944F/R and KAN-AGE F/R primers.

**Creation of the *B. melitensis* 16MΔE Mutant**

For the production of a *B. melitensis* 16M Region E mutant via homologous recombination, a Region E-Kanamycin-Region E (E-KAN-E) fragment was produced by *Eco*R I digestion of pUC19ΔE. Successful digestion of the plasmid was confirmed via gel electrophoresis (Figure 13). The 1,640 base pair E-KAN-E fragment was electroporated into *B. melitensis* 16M and screened for homologous recombination using SBA plates supplemented with 45 µg/ml kanamycin. Genomic DNA isolation was performed on colonies displaying ampicillin and kanamycin resistance. PCR of the isolated DNA using the ORF-944F/R primers revealed the presence of the 1.6 kilobase E-KAN-E fragment. This integration of E-KAN-E into the chromosome was visualized by gel electrophoresis (Figure 14). The resulting mutant was named *B. melitensis* 16MΔE.

**Colonization of *B. abortus* 2308-QAE and *B. melitensis* 16MΔE**

A short term colonization study was preformed to see if *B. abortus* 2308-QAE or *B. melitensis* 16MΔE could colonize non-pregnant goats. Twenty-four goats divided into four equal groups were inoculated with either $1x10^9$ cfu/ml of *B. abortus* 2308, *B. abortus* 2308-QAE, or *B. melitensis* 16M or $1x10^{10}$ cfu/ml of *B. melitensis* 16MΔE.

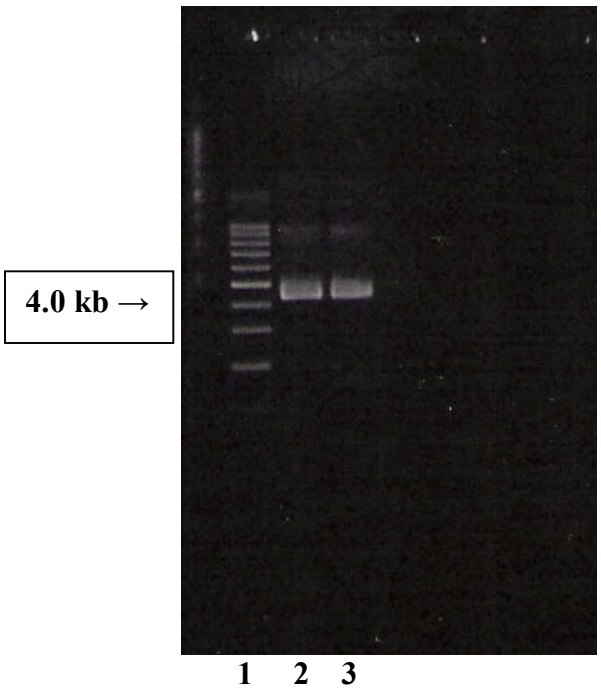


**Figure 11.** Agarose gel electrophoresis of pUC19ΔE miniprep DNA. The arrow indicates a 4.0 bp band on the molecular weight ladder.

Lane 1 - Supercoiled DNA Molecular Weight Ladder
Lanes 2-3 - pUC19ΔE DNA (4,326 bp)

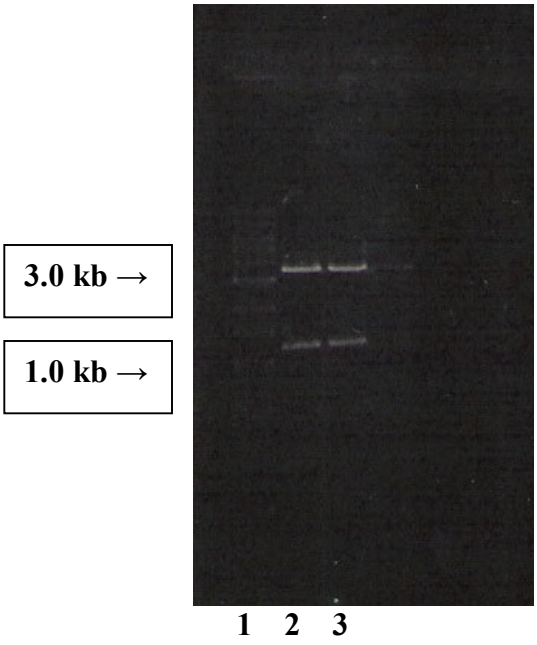


**Figure 12.** Agarose gel electrophoresis of pUC19ΔE digested with *Age* I. Digestion releases a 1,200 bp interior Region E fragment and linearizes a 3,486 bp pUC19+E vector. The arrows indicate 1.0 and 3.0 kb bands on the molecular weight ladder.

Lane1 - 1 kb Molecular Weight Ladder
Lanes 2-3 - *Age* I-digested pUC19ΔE

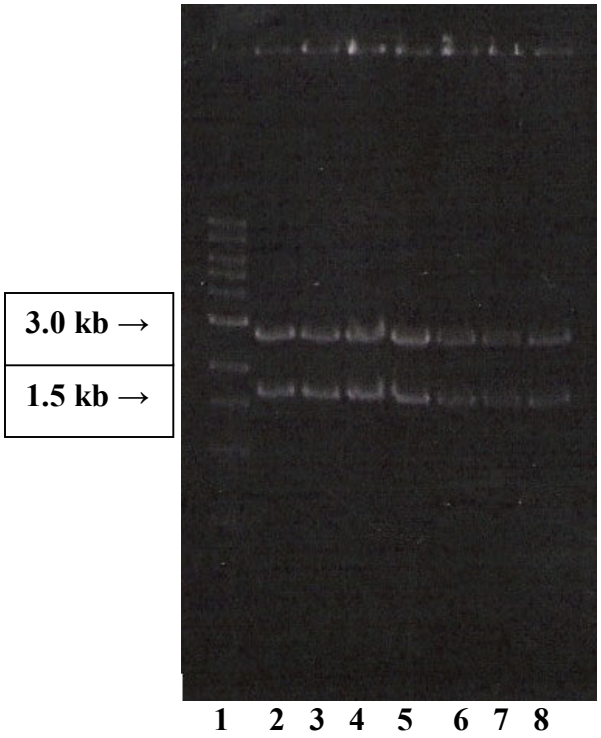


**Figure 13.** Agarose gel electrophoresis of pUC19ΔE digested with *Eco*R I. Digestion releases a 1,640 E-KAN-E fragment and linearizes a 2,678 pUC19 vector. The arrows indicate 1.5 and 3.0 kb bands on the molecular weight ladder.

Lane 1 - 1 kb Molecular Weight Ladder
Lanes 2-8 - *Eco*R I-digested pUC19ΔE

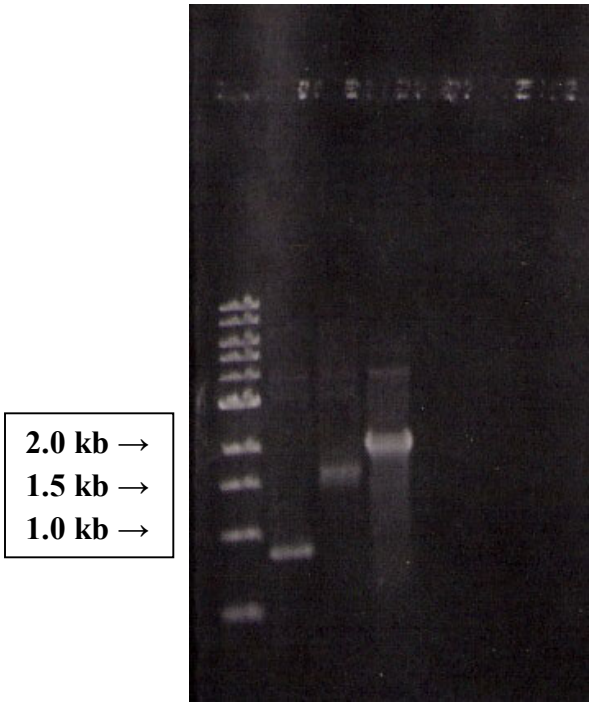


**Figure 14.** Agarose gel electrophoresis of PCR-amplified products from DNA isolated from *B. melitensis* 16MΔE mutants using the KAN-AL and KAN-AR and ORF-944F and ORF-944R primers. The arrows indicate 1.0, 1.5, and 2.0 kb bands on the molecular weight ladder.

Lane 1 - 1 kb Molecular Weight Ladder
Lane 2 - *B. melitensis* 16MΔE DNA with KAN-AL and KAN-AR primers (840 bp)
Lane 3 - *B. melitensis* 16MΔE DNA with ORF-944F and ORF-944R primers (1,640 bp)
Lane 4 - *B. melitensis* 16M with ORF-944F and ORF-944R primers (2,000 bp)

Greater numbers of the genetically-manipulated *B. melitensis* 16M strain than the virulent parental strain was used to establish initial colonization of the lymphoid tissue in the event that the disruption mutant was severely attenuated (Elzer et al 2002). Two animals from each group were sacrificed at days 7, 14, and 21. The following tissues were collected and examined bacteriologically: parotid, prescapular, internal iliac, inguinal, and supramammary lymph nodes; liver; and spleen. Results were recorded as colony-forming-units pre gram of tissue. Colonization results are presented in Table 1.

All resulting colonies were evaluated to verify their *Brucella* origin via oxidase, catalase, and urease tests. Both *B. melitensis* 16ΔM and *B. abortus* 2308-QAE were also plated on SBA plates supplemented with either 45 µg/ml kanamycin or 100 µg/ml ampicillin, respectively. All colonies were confirmed to be *Brucella* and grew on the appropriate antibiotic-supplemented media.

Serological analysis of the colonization goats at days 7, 14, and 21 via *Brucella* Card Test and western immunoblot analysis revealed that all animals at days 14 and 21 were seropositive. At day 7, animals inoculated with *B. abortus* 2308, *B. abortus* 2308-QAE, and *B. melitensis* 16M were seronegative; whereas, animals infected with *B. melitensis* 16ΔM were seropositive (Table 2).

**Pathogenesis of *B. abortus* 2308-QAE and *B. melitensis* 16MΔE in Pregnant Goats**

To assess the pathogenicity of *B. melitensis* 16MΔE and *B. abortus* 2308-QAE in the ruminant host, pregnant goats in late-gestation were exposed to either the virulent parental strains, *B. melitensis* 16M or *B. abortus* 2308; the variant, *B. abortus* 2308-QAE; or the mutant, *B. melitensis* 16MΔE. Pregnant, mixed-breed goats were inoculated conjunctively with $1x10^7$ cfu/ml of inoculum. Study results are presented in Table 3.

**Table 1.** Colonization of non-pregnant goats inoculated with *B. abortus* 2308, *B. abortus* 2308-QAE, *B. melitensis* 16M, or *B. melitensis* 16MΔE.

| *Brucella* | Days Post Infection | Parotid Lymph Node | Prescapular Lymph Node | Liver | Spleen | Internal Iliac Lymph Node | Inguinal Lymph Node | Supramammary Lymph Node |
|---|---|---|---|---|---|---|---|---|
| 2308 | 7 | 2/2 (77.5)* | NEG[§] | NEG | NEG | NEG | NEG | NA[a] |
| QAE | 7 | 2/2 (148.5) | NEG | NEG | NEG | NEG | NEG | NA |
| 16M | 7 | 2/2 (392.0) | NEG | 1/2 (1) | 1/2 (6.0) | NEG | NEG | NA |
| 16ΔM | 7 | 2/2 (379.0) | 2/2 (1.5) | NEG | 1/2 (7.0) | 1/2 (0.5) | 1/2 (0.5) | NEG |
| | | | | | | | | |
| 2308 | 14 | 2/2 (96.5) | 2/2 (31.0) | NEG | 2/2 (48.5) | 1/2 (1.0) | 2/2 (5.5) | NA |
| QAE | 14 | 2/2 (537.0) | 1/2 (0.5) | NEG | 2/2 (3.0) | 1/2 (0.5) | NEG | NA |
| 16M | 14 | 2/2 (373.5) | 2/2 (5.0) | 2/2 (8) | 2/2 (80.0) | 1/2 (19.0) | 1/2 (0.5) | NA |
| 16ΔM | 14 | 2/2 (168.5) | 2/2 (170.5) | 2/2 (125) | 2/2 (524.0) | 1/2 (113.0) | 2/2 (175.0) | NA |
| | | | | | | | | |
| 2308 | 21 | 2/2 (43.0) | NEG | NEG | 2/2 (3.0) | NEG | NEG | NA |
| QAE | 21 | 1/2 (239.5) | 1/2 (31.5) | 1/2 (0.5) | 1/2 (11.0) | 1/2 (1.0) | NEG | NA |
| 16M | 21 | 2/2 (133.0) | 1/2 (3.0) | 1/2 (2) | 2/2 (13.5) | NEG | NEG | NA |
| 16ΔM | 21 | 2/2 (155.5) | 2/2 (6.5) | 1/2 (1.5) | 2/2 (68.5) | 2/2 (2.0) | 2/2 (2.5) | NA |

* Calculated in mean CFU/gram of tissue.
[§] Negative.
[a] Not applicable.

**Table 2.** Serological analysis of non-pregnant colonization goats inoculated with *B. abortus* 2308, *B. abortus* 2308-QAE, *B. melitensis* 16M, or *B. melitensis* 16MΔE.

| ***Brucella*** | **Day 7*** | **Day 14*** | **Day 21*** |
|---|---|---|---|
| *B. abortus* 2308 | 2/2 NEG | 2/2 POS | 2/2 POS |
| *B. abortus* 2308-QAE | 2/2 NEG | 2/2 POS | 2/2 POS |
| *B. melitensis* 16M | 2/2 NEG | 2/2 POS | 2/2 POS |
| *B. melitensis* 16MΔE | 2/2 POS | 2/2 POS | 2/2 POS |

*Evaluated using the Brucella Card Test and western immunoblot analysis using cell lysates from *B. abortus* 2308, *B. abortus* 2308-QAE, *B. melitensis* 16M, or *B. melitensis* 16MΔE.

**Table 3.** Colonization and abortion rates of pregnant goats inoculated with *B. abortus* 2308, *B. abortus* 2308-QAE, *B. melitensis* 16M, or *B. melitensis* 16MΔE.

| | ***B. abortus* 2308** | ***B. abortus* 2308-QAE** | ***B. melitensis* 16M** | ***B. melitensis* 16MΔE** |
|---|---|---|---|---|
| **Dam/Kid Pair* Bacterial Colonization Rate** | 5/11<br>45%** | 9/12<br>75% | 11/14<br>79% | 6/6<br>100% |
| **Abortion Rate (Aborted fetuses/weak kids)** | 27%** | 67% | 78% | 50% |

*One positive dam or kid constituted a positive dam/kid pair. Dam tissues homogenized and plated included: parotid, prescapular, and supramammary lymph nodes; liver; spleen; internal iliac; and mammary gland. Kid tissues included: lung and abomasal fluid.
**$p \leq 0.05$.

Eleven goats inoculated with *B. abortus* 2308 resulted in a 27% abortion rate, as compared to, 12 goats infected with *B. abortus* 2308-QAE exhibiting a 67% abortion rate ($P<0.05$). Additionally, a 78% abortion rate was observed in 14 goats inoculated with *B. melitensis* 16M; whereas, only a 50% abortion rate was recorded in seven goats infected with the *B. melitensis* 16MΔE disruption mutant.

Dam and kid culture results were analyzed in pairs. A positive kid or dam within a pair was recorded as a culture positive pair (Table 3). The parotid, prescapular, internal iliac, and supramammary lymph nodes; liver; spleen; and mammary glands were taken from the adults. Lung and abomasal fluid was taken from the fetuses or kids. All tissues were homogenized and plated for bacterial growth. Results were recorded as colony-forming-units pre gram of tissue. Bacteriologically, five of 11 goats (45%) infected with *B. abortus* 2308 were culture positive. Nine of 12 goats (75%) inoculated with *B. abortus* 2308-QAE were found to be culture positive. *B. melitensis* 16M infected goats resulted in 11 of 14 (79%) animals being culture positive. All seven (100%) of the pregnant goats infected with *B. melitensis* 16ΔM were culture positive at necropsy or time of death (Table 3).

All emerging colonies were evaluated via oxidase, catalase, and urease tests to verify their *Brucella* origin. The mutant, *B. melitensis* 16ΔM, and variant, *B. abortus* 2308-QAE, colonies were additionally plated on SBA plates supplemented with either 45 µg/ml kanamycin or 100 µg/ml ampicillin, respectively. All colonies were confirmed to be *Brucella* and grew on the appropriate antibiotic-supplemented media.

**Serological Responses to *B. abortus* 2308-QAE and *B. melitensis* 16MΔE**

Serological responses of pregnant goats to *B. melitensis* 16M, *B. melitensis* 16MΔE, *B. abortus* 2308, and *B. abortus* 2308-QAE were determined via Brucellosis Card Test and western immunoblot analysis (Figure 15) using cell lysates from each of the aforementioned test strains. At termination, serological analyses of all necropsied, inoculated animals were found to be positive for the presence of *Brucella* antibodies (Table 4).

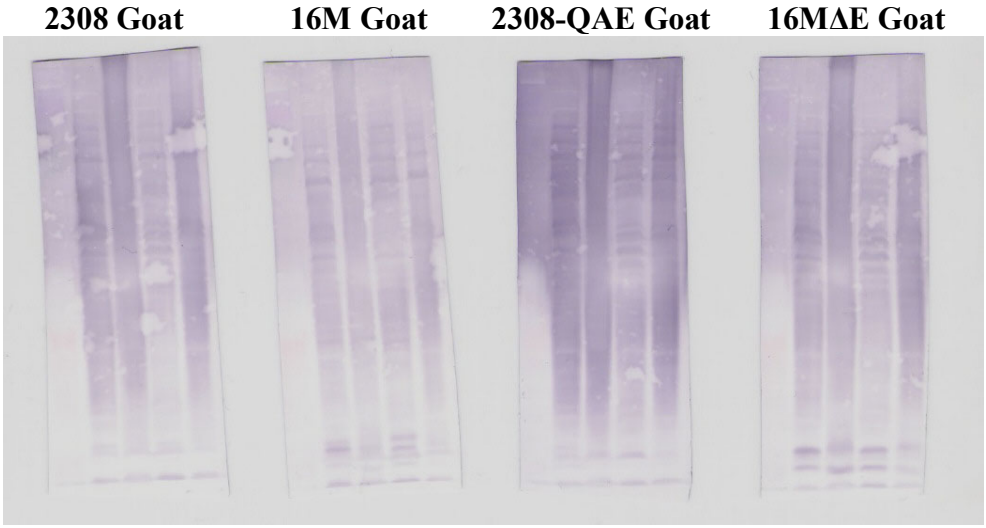


**Figure 15.** Western immunoblot analysis of *B. abortus* 2308, *B. melitensis* 16M, *B. abortus* 2308-QAE, or *B. melitensis* 16MΔE-infected goats. Sera were taken at necropsy, and the blots were run using cell lysates from each of the aforementioned strains.

Lane 1 - Kaleidoscope Pre-stained Standard
Lane 2 - *B. abortus* 2308 Cell Lysates
Lane 3 - *B. abortus* 2308-QAE Cell Lysates
Lane 4 - *B. melitensis* 16M Cell Lysates
Lane 5 - *B. melitensis* 16MΔE Cell Lysates

**Table 4**. Serological analysis of pregnant goats inoculated with *B. abortus* 2308, *B. abortus* 2308-QAE, *B. melitensis* 16M, or *B. melitensis* 16MΔE.

| | ***B. abortus* 2308** | ***B. abortus* 2308-QAE** | ***B. melitensis* 16M** | ***B. melitensis* 16MΔE** |
|---|---|---|---|---|
| **Serologic Analysis* via Card Test and Immunoblot (+ Response)** | 10/11<br>91% | 12/12<br>100% | 11/14<br>79% | 6/7<br>86% |

*One *B. abortus* 2308, three *B. melitensis* 16M, and one *B. melitensis* 16MΔE -inoculated goat(s) died prior to necropsy, therefore, no post-inoculation sera was collected on these animals. Western blot analysis used cell lysates from *B. abortus* 2308, *B. abortus* 2308-QAE, *B. melitensis* 16M, or *B. melitensis* 16MΔE.

## DISCUSSION

An important step in understanding the molecular basis of pathogenesis is the identification of genes utilized in causing disease. Opportunities to determine the possible virulence genes in the *Brucella* genus increased with the complete characterization of genomes within the genus. Various techniques have been used to evaluate potential virulence genes (Hensel and Holden 1996).

Transposon mutagenesis has been well documented for the study of possible virulence genes (de Lorenzo et al 1990). Mini-Tn5 has been used to identify virulence factors including a two-component system, BvrR-BvrS, critical for intracellular growth in *B. abortus* (Sola-Landa et al 1998). A type IV secretion system was also identified by screening a Tn*Bla*M library of *B. suis* for growth in HeLa cells (O'Callahan et al 1999).

Signature-tagged mutagenesis (STM) has also allowed for the screening of numerous transpositional mutants in a single animal (Hensel et al 1995) and has been successfully applied to the identification of attenuated mutants in Gram-negative bacteria (Darwin and Miller 1999; Edelstein et al 1999). This technique has also been used to identify numerous *Brucella* virulence and survival genes (Lestrate et al 2000; Hong et al 2000; Kahl-McDonagh and Ficht 2006; Zygmunt et al 2006; Wu et al 2006).

Many experiments have been conducted using deletion mutants generated by gene replacement via homologous recombination to identify gene function (Halling et al 1991; Drazek et al 1995; Elzer et al 1994; Gee et al 2004 and 2005). Edmonds et al (2002a and 2002b) described a *B. melitensis* 16M OMP 25 deletion mutant created via gene replacement, which colonized fewer pregnant goats and kids than the virulent *B.*

*melitensis* 16M strain without resulting in abortions. Experiments such as these will lead to the discovery of potential efficacious vaccine candidates.

A recent study by del C. Rocha-Gracia et al (2002) investigated the ability of various strains of *B. abortus* and *B. melitensis* to hemagglutinate human and animal red blood cells. It identified a 29 kilo Dalton surface protein (SP29), which is associated with the hemagglutination of all the *Brucella* strains tested with human (A+ and B+) and animal (rabbit, hamster, rat, and mouse) red blood cells.

This current study suggested that the manipulation of a *B. melitensis* 16M putative hemagglutinin gene, Region E, would play a role in the virulence of the organism. The absence of this hemagglutinin gene in *B. abortus* 2308 raised the question of whether or not the gene has an effect on the colonization and pathogenesis of either *B. melitensis* 16M and *B. abortus* 2308 in the caprine model. It was proposed that the expression of Region E *in trans* in *B. abortus* 2308 would cause increased virulence. In contrast, it was also proposed that a *B. melitensis* 16M Region E disruption mutant created via gene replacement and homologous recombination would be attenuated in the caprine model. The caprine model was used to test the virulence of the *B. melitensis* 16M Region E disruption mutant and the *B. abortus* 2308 region E variant based upon colonization of pregnant, non-pregnant females and fetuses/kids, and delivery status of the fetuses/kids (Elzer et al 2002). *B. melitensis* 16MΔE and *B. abortus* 2308-QAE were evaluated for their ability to colonize the expected reticuloendothelial organs and cause abortions in goats.

Animals inoculated conjunctively are usually colonized in the parotid lymph node within the first three days post-infection with the organism disseminating to the liver and

spleen by seven days post-infection. The supramammary and internal iliac lymph nodes should show signs of colonization by 14 days post-infection (Elzer et al 2002).

*B. abortus* 2308-QAE displayed colonization results typical of virulent *Brucella* species in that the parotid lymph node was colonized by seven days post-inoculation. However, *B. abortus* 2308-QAE infection resulted in a greater number of colony forming units per gram of tissue (cfu/gm) than its parental *B. abortus* 2308 strain in animals given the same dosage of infectious organisms, $1x10^9$ cfu/ml (Table 1). There was no growth seen in the liver or spleen which was in contrast to minimal numbers seen in *B. melitensis* 16M inoculated animals. By day 14 post-inoculation, *B. abortus* 2308-QAE was cultured from the parotid, prescapular, and internal iliac lymph nodes and the spleen. *B. abortus* 2308 was cultured from all tissues except the liver with possible evidence of clearance of the organism by day 21 post-inoculation. Day 21 data revealed that *B. abortus* 2308-QAE, similar to what was seen in *B. melitensis* 16M, could still be found in every tissue sample tested except the inguinal lymph node with an increased number of cfu/gm in comparison to *B. abortus* 2308.

Serologically, all animals infected with *B. abortus* 2308-QAE tested positive for the presence of *Brucella* antibodies in their sera on the Brucellosis Card Test and by western immunoblot analysis, which used cell lysates from *B. abortus* 2308, *B. abortus* 2308-QAE and *B. melitensis* 16M (Table 2). Western blot analysis results were indicative of what is typically seen when the sera from animals exposed to smooth *Brucella* species are analyzed (Figure 15).

*B. melitensis* 16MΔE, in comparison to *B. melitensis* 16M, colonized the animals to a greater extent by seven days post-infection with the parotid, prescapular, internal

iliac, and inguinal lymph nodes as well as the spleen being colonized by the mutant with similar cfu/gm of tissue as *B. melitensis* 16M (Table 1). By days 14 and 21 post-inoculation, all tissues from *B. melitensis* 16MΔE-infected animals had been colonized very similarly to those exposed to the *B. melitensis* 16M parental strain. Animals exposed to the *B. melitensis* 16MΔE disruption mutant were given a log higher infectious dose at $1x10^{10}$ cfu/ml than the *B. melitensis* 16M-infected animals, which received $1x10^{9}$ cfu/ml. A higher dosage of the genetically-manipulated strain, *B. melitensis* 16MΔE, was used to establish initial colonization of the lymphoid tissue in the event that the mutant was severely attenuated (Elzer et al 2002). All animals were found to be seropositive at necropsy via the Brucellosis Card Test and western blot immunoblot analysis using cell lysates from *B. abortus* 2308, *B. abortus* 2308-QAE, *B. melitensis* 16M, and *B. melitensis* 16MΔE (Table 2).

This short-term colonization study revealed no sign of attenuation in *B. melitensis* 16MΔE's ability to invade the mucosal epithelium, infect the animal, and disseminate throughout the animal's body. A similar rate and quantity of cfu/gm of tissue as its virulent parental strain, *B. melitensis* 16M, was also observed.

*B. abortus* 2308-QAE, the *B. abortus* 2308 variant, was also capable of infecting and colonizing the animal with no sign of attenuation. There was a slight increase in the number of cfu/gm of tissues colonized by the organism in comparison to its virulent parental strain.

Typically, pathogenesis studies in the goat model reveal a 90-100% dam-kid pair colonization and a 70-100% abortion rate with *B. melitensis* 16M-infected animals. In contrast, studies with animals infected with *B. abortus* 2308 usually display a 50-70%

dam-kid pair colonization and a 30-50% abortion rate. The pathogenicity of mutants or variants is measured by comparing their colonization and abortion rates to those of their virulent parental strains. Mutant strains can exhibit no statistical difference from the parental strains or result in up to 50% colonization or 0-20% abortion rates (Elzer et al 2002).

In this pathogenesis study, animals infected with *B. abortus* 2308-QAE aborted (67%) and were colonized (75%) at rates more similar to the 70-100% abortion and 90-100% colonization rates of *B. melitensis* 16M (Table 3). Statistically, these *B. abortus* 2308-QAE rates were significantly different from the *B. abortus* 2308 parental strain rates observed ($p \leq 0.05$). Results showed a 45% colonization and a 27% abortion rate in *B. abortus* 2308-infected pregnant goats, which is consistent with the reported colonization and abortion rates of 50-70% and 30-50%, respectively. The addition and expression of Region E, *in trans*, in *B. abortus* 2308 caused a significant increase in the pathogenicity in *B. abortus* 2308-QAE-infected pregnant goats ($p \leq 0.05$). However, *B. abortus* 2308-QAE appeared less pathogenic than *B. melitensis*16M, causing fewer abortions and lower colonization rates.

Animals infected with *B. melitensis* 16MΔE showed a 30% reduction in abortions when compared to the 70-100% abortion rates typically seen in pregnant goats infected with *B. melitensis* 16M. Data also revealed a 28% reduction when comparing the abortion rate of *B. melitensis* 16MΔE-infected goats to the 78% rate of *B. melitensis* 16M-infected goats seen in this study (Table 3). This reduction could indicate attenuation of the mutant's ability to cause abortions in pregnant goats. All dams (100%) and six of twelve kids/fetuses (50%) infected with *B. melitensis* 16MΔE were culture

positive at necropsy or time of death, demonstrating the disruption mutant's ability to effectively colonize both the dam and the gravid uterus (Table 3).

All animals infected in the pathogenesis study were found to have a positive serologic response typical of a smooth *Brucella* strain infection (Table 4) (Figure 15). Some animals died before necropsy and were unable to be analyzed serologically. One *B. abortus* 2308, three *B. melitensis* 16M, and one *B. melitensis* 16MΔE -inoculated goat(s) died prior to necropsy, therefore, no post-inoculation sera was collected on these animals. These deaths were not believed to be attributed to the *Brucella* infections but to the birthing process or other unrelated circumstances.

Region E is purported to be a putative hemagglutinin and may be a virulence factor for *B. melitensis* 16M. It may also be evaluated as a possible host specificity factor for *B. melitensis* 16M in goats.

Further studies should be conducted to determine the possibility of Region E being a host specificity factor by evaluating its effects in other animals. Additionally, the immunological response elicited by Region E must be assessed. Studies should also be done to exploit the stability of pQAE in other *Brucella* species or strains to be tested as possible vaccine candidates given the increased colonization rate of pQAE-containing *B. abortus* 2308. Placing this plasmid into a rough *Brucella* strain or current vaccine may be effective in developing an efficacious vaccine that spans the *Brucella* genus to help eradicate the worldwide problem of brucellosis for both man and animals.

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## Vita

Quinesha Laticia Perry is a native of Port Allen, Louisiana. She received her high school diploma on Friday, May 20, 1994, from Brusly High School in Brusly, Louisiana. On Saturday, May 9, 1998, she obtained a Bachelor of Science degree in biology from Xavier University of Louisiana in New Orleans. She will always love New Orleans, and it will always be her home-away-from-home! Quinesha received a Master of Science degree in microbiology from Louisiana State University in Baton Rouge, Louisiana, on Friday, December 21, 2001. In January 2002, she enrolled in the Veterinary Medical Sciences program in the Department of Pathobiological Sciences at the Louisiana State University School of Veterinary Medicine in Baton Rouge, Louisiana, and is currently a candidate for the degree of Doctor of Philosophy. Quinesha is thoroughly elated at this accomplishment; but above all, the one thing that she would like everyone to know, recognize, and remember her as is a child of God redeemed by the blood of Jesus.

*"But seek ye first the kingdom of God,*
*and His righteousness; and all these things*
*shall be added unto you."*
*~Matthew 6:33*