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Evaluating the virulence of a *Brucella melitensis* hemagglutinin gene in the caprine model

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ABSTRACT

With the completion of the genomic sequence of *Brucella melitensis* 16M, a putative hemagglutinin gene was identified which is present in 16M and absent in *Brucella abortus*. The possibility of this hemagglutinin being a potential virulence factor was evaluated via gene replacement in *B. melitensis* yielding 16MΔE and expression *in trans* in *B. abortus* 2308-QAE.

Utilizing the caprine brucellosis model, colonization and pathogenesis studies were performed to evaluate these strains. *B. melitensis* 16M hemagglutinin gene expression *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 *B. melitensis* 16M virulence in pregnant goats. The *B. melitensis* disruption mutant's colonization and abortion rates demonstrated no attenuation in colonization but displayed a 28% reduction in abortions when compared to parental *B. melitensis* 16M.

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1. Introduction

Brucella species are short, non-motile, non-sporulating, non-encapsulated, Gram-negative aerobic rods. They are facultative intracellular pathogens of animals and humans [1]. The *Brucella* genus is highly homogeneous with all members showing greater than 90% homology in DNA–DNA pairing studies [2,3], and little is known about *Brucella* virulence. The genus *Brucella* consists of six classical species, each with a preference for a primary host and varying degrees of pathogenicity. *Brucella melitensis* primarily infects goats and is the most pathogenic for humans; *Brucella abortus* infects cattle.

Brucella LPS has important cell surface properties yet there is no evidence showing its role in invasion [4]. Other outer membrane proteins may also play a role in the organisms' virulence [5,6]. An organism's ability to adhere to a mucosal surface is a crucial first step in the pathogenesis of many pathogens [7]. Initial attachment of the brucellae to epithelial is mostly unknown. With the completion of *Brucella* species entire genomes, specifically *B. melitensis* 16M and *B. abortus*, studies have been and are currently underway to detect and characterize novel genes that may be involved in *Brucella* pathogenicity [8,9]. Of particular note is a putative hemagglutinin gene found within the *B. melitensis* 16M genome that is absent in *B. abortus* [9,10]. The gene is present in *Brucella suis* and *Brucella canis* but with minor nucleotide substitutions. There are two copies of the gene in *Brucella 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 cell wall surface proteins of *B. suis*, *B. ovis* and *B. canis* [11].

A study done by del C Rocha-Gracia et al. [5] 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. All *Brucella* strains tested (*B. abortus* 2308, *B. abortus* S19, *B. abortus* 02, and *B. melitensis* 03) showed hemagglutination to the RBCs from the various sources, with *B. melitensis* 03 showing the highest hemagglutination titers against all red cells and *B. abortus* 2308 the lowest titer.

This study evaluated the virulence of Region E, a putative ~2.0 kilobase (kb) hemagglutinin gene using the completed genome of *B. melitensis* 16M [8]. Experiments using a Region E disruption mutant of *B. melitensis* 16 M and a variant of *B. abortus* 2308 expressing Region E *in trans* were carried out in the caprine brucellosis model to provide insight into molecular basis of *Brucella* virulence, host specificity, and possible vaccine development.

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2. Materials and methods

2.1. Bacterial strains

Work with *Brucella* spp. was approved by the university IBRDSC under a Select Agent registration with the CDC. Virulent *B. abortus* strain 2308 and *B. melitensis* strain 16M were used in these studies to create *B. abortus* 2308-QAE and *B. melitensis* 16MΔE. Parental virulent strains as well as test strains were cultured from the laboratory stocks when needed. Glycerol stocks of *B. abortus* 2308 and *B. melitensis* 16M were grown on Schaedler Brucella Agar (SBA) (Difco Laboratories, Detroit, MI, U.S.A.) and *B. abortus* 2308-QAE and *B. melitensis* 16MΔE were grown on SBA containing 100 μg/ml ampicillin or 45 μg/ml kanamycin, respectively. Plates were incubated at 37 °C in a 5% CO_2 atmosphere for 2–3 days.

Inoculation doses of *B. abortus* 2308, *B. melitensis* 16M, *B. abortus* 2308-QAE, and *B. melitensis* 16MΔE were made as previously described [12]. 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 the day of use.

2.2. Creation of B. abortus 2308 variant

A 4950 bp plasmid, pBBR1MCS-4 [13] was digested using *Eco*R V (New England Biolabs, Beverly, MA, U.S.A.). A ∼2.0 kb PCR-amplified putative hemagglutinin gene, Region E, was generated from *B. melitensis* 16M genomic DNA using the primers ORF-944F (5′-GAATTGGCGACCTGACTGAGGA-3′) and ORF-944R (5′-CTCACGGCTGTTCTCCTTTAACA-3′) (The Institute of Molecular Biology and Medicine at the University of Scranton, Scranton, PA, U.S.A.). 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, Madison, WI, U.S.A.) to create pQAE. The ligation mixture was then used to transform One Shot® Chemically Competent Cells according to the manufacturer's directions (Invitrogen Corporation, Carlsbad, CA, U.S.A.). Successful transformants were cultured and their plasmids isolated using the Qiagen Buffer System (Qiagen Inc., Valencia, CA, U.S.A.). The isolated plasmid DNA was electroporated into *B. abortus* 2308 as previously described [12], creating *B. abortus* 2308-QAE.

2.3. Confirmation of B. abortus 2308 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. 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. The new variant of *B. abortus* 2308 was named *B. abortus* 2308-QAE. Presence of the gene was confirmed via PCR amplification of the putative hemagglutinin using the Region E primers and restriction enzyme digestion of pQAE.

2.4. Creation of B. melitensis 16M mutant

A ∼2.0 kb PCR-amplified Region E fragment was generated from *B. melitensis* 16M genomic DNA using the Region R primers, ORF-944F and ORF-944R, as described above. The PCR fragment was then ligated into the linear 3519 bp pCR®-Blunt II-TOPO® vector following the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA, U.S.A.) using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen Corporation). The cloning reaction was then used in the TOPO® Cloning and Transformation protocol with One Shot® Chemically Competent Cells according to the corporation protocol (Invitrogen Corporation) and plated on LB agar plates supplemented with 45 μg/ml kanamycin and incubated overnight at 37 °C. Successful transformants were cultured and their plasmids isolated for confirmation of pTOPO + E via PCR using Region E primers.

Region E was then excised from the TOPO® vector via *Eco*R I (New England Biolabs) endonuclease restriction digestion. 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. The ligation mixture was then used to transform One Shot® Chemically Competent Cells and plated on LB agar plates supplemented with 100 μg/ml ampicillin and incubated overnight at 37 °C. Successful transformants were cultured and their plasmids isolated for confirmation of pUC19E, a 4686 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 [13] using the primers KAN-AL (5′-TGACCGGTTCATTTCGAACCCCAGAGTC-3′) and KAN-AR (5′-AGACCGGTACAGGATGAGGATCGTTTCG-3′), with the *Age* I restriction endonuclease sequence, 5′-ACCGGT-3′, added to their 5′ ends. Both pUC19E and the PCR-amplified kanamycin antibiotic cassette (KAN) were digested with *Age* I. *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 new plasmid pUC19ΔE was digested with *Eco*R I to produce the 1640 Region E-Kanamycin-Region E (E-KAN-E) fragment. The fragment was electroporated into *B. melitensis* 16M as previously described [12], creating *B. melitensis* 16MΔE.

2.5. Confirmation of B. melitensis 16M mutant

Genomic DNA was isolated from *B. melitensis* 16MΔE colonies displaying kanamycin resistance. PCR amplification of the isolated DNA using the ORF-944F/R primers revealed the presence of the 1.6 kilobase E-KAN-E fragment. The integration of E-KAN-E into the chromosome was visualized by gel electrophoresis.

2.6. Standard identification tests

Potential variant/mutant colonies were isolated for *Brucella* typing using techniques commonly performed to differentiate *Brucella* spp. from other Gram-negative organisms. Standard biochemical tests were performed, including urease, oxidase, and catalase, along with observing colony morphology and growth rate [14]. Suspected variants/mutants along with their parental strains were also tested for sensitivity to dyes: azure A, basic fuchsin, crystal violet, pyronin, safranin, and thionin, according to the manufacturer's protocol (Key Scientific Products, Round Rock, TX, U.S.A.).

2.7. Goats

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

For the colonization studies, 24 adult Spanish goats were obtained from commercial herds or from the LSU herd (Louisiana State University Agricultural Center, Baton Rouge, LA, U.S.A.). At predetermined time points the goats were euthanized by captive-bolt and exsanguination. The following tissues were collected: parotid, prescapular, internal iliac, supramammary or inguinal lymph nodes; liver; and spleen.

For the pathogenesis studies, 37 pregnant Angora and/or Spanish goats and eight sexually-mature, non-pregnant female Spanish

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