Attenuation of a *Brucella abortus* mutant lacking a major 25 kDa outer membrane protein in cattle

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Objective—To determine the virulence of a *Brucella abortus* mutant, BA25, lacking a major 25 kd outer membrane protein (*Omp25*) in cattle.

Animals—20 mixed-breed heifers in late gestation.

Procedure—10 heifers were inoculated with 1 X 10^7 colony-forming units of the *Omp25* mutant via the conjunctival sac, and an equal number were infected with the virulent parental strain *B abortus* 2308. The delivery status of the dams was recorded, and colonization was assessed following necropsy. The ability of BA25 to replicate inside bovine phagocytes and chorionic trophoblasts was also evaluated in vitro because of the propensity of virulent brucellae to replicate inside these cells in vivo.

Results—The parental strain induced abortions in 5 of 10 inoculated cattle, whereas only 1 of 10 dams exposed to BA25 aborted. *Brucella abortus* strain 2308 colonized all of the cow-calf pairs and induced *Brucella*-specific antibodies in 100% of the dams. In contrast, BA25 was isolated by bacteriologic cultural technique from 30% of the calves and 50% of the inoculated dams (n = 10). Of the 10 heifers inoculated with BA25, 4 did not develop *Brucella*-specific antibodies nor were they colonized by the mutant strain. In bovine macrophages and chorionic trophoblasts, BA25 replicated in significantly lower numbers than the virulent parental strain (n = 3).

Conclusions and Clinical Relevance—The 25 kd outer membrane protein may be an important virulence factor for *B abortus* in cattle. The attenuation of the *Omp25* mutant in cattle may involve the inability of BA25 to replicate efficiently in bovine phagocytes and chorionic trophoblasts.(*Am J Vet Res* 2001;62:1461–1466)

Brucellosis, or Bang's disease, is a serious economic threat to beef and dairy cattle producers throughout much of the world. The etiologic agent, *Brucella abortus*, is a gram-negative facultative intracellular pathogen that causes cattle to abort during the last trimester of pregnancy.[1,2] *Brucella abortus* is also pathogenic for man, causing a variety of possible signs including intermittent fever, arthritis, osteomyelitis, and spondylitis.[3]

This pathogen gains entry to the host via the mucosal surfaces and is phagocytized by macrophages and neutrophils in an effort by the host to eliminate the organism.[1,2] However, once inside the phagocyte, *B abortus* is able to survive and replicate.[4,5] The phagocyte migrates via the lymphatic system to the draining lymph node, where brucellae infection causes cell lysis and eventual lymph node hemorrhage approximately 2 to 3 weeks following exposure.[1] Because of vascular damage, some of the bacteria enter the bloodstream, and subsequent bacteremia develops, which results in dissemination of the pathogen throughout the host.[1,2]

If the infected animal is pregnant, *B abortus* will colonize and replicate to high numbers in the chorionic trophoblasts of the developing fetus.[1,2] The resulting tissue necrosis of the fetal membranes allows transmission of the bacteria to the fetus. The net effect of chorionic and fetal colonization is the premature delivery of a dead calf during the last trimester of pregnancy. The predilection of *B abortus* for late-gestational chorionic trophoblasts has not been fully explained. Evidence does exist that hormones and possibly the sugar erythritol may play an important role in the tropism of brucellae to the reproductive tract.[1,6] Late-gestational chorionic trophoblasts, compared with trophoblasts obtained from early-gestational placentas, produce higher amounts of erythritol and different concentrations of hormones.[1] *Brucella abortus* is also known to invade and replicate poorly in early-gestational trophoblasts, compared with late-gestational cells.[7] The predilection of *B abortus* to invade and replicate inside bovine trophoblasts is an important step in the abortion process.

Three major groups of **outer membrane proteins (Omp)** have been identified in *Brucella* spp.[8,9] The group-1 Omp (94 kd) are considered minor components of the outer membrane, whereas the group-2 Omp (41 to 43 kd) are functional porins.[9,10] The group-3 Omp range from 25 to 31 kd and consist of 2 different **proteins of 25 (Omp25)** and **31 kd (Omp31)**, respectively.[9,11] Unlike the other *Brucella* spp, *B abortus* lacks Omp31 because of a chromosomal deletion.[12,13] Although no function has been described for Omp25, the *omp25* gene has been sequenced and cloned from *B*

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abortus.[14] The Omp25 protein is highly conserved throughout the *Brucella* genus.[11] This protein is bound by a possible covalent bond to the peptidoglycan layer of the cell envelope and is expressed on the outer membrane in association with lipopolysaccharide.[15,16]

Brucella abortus strain 2308 is a virulent challenge strain used by several different laboratories that is documented to cause abortions in pregnant cattle.[1,17,18] To determine the role of Omp25 in virulence, a mutant of *B abortus* strain 2308 containing a disruption in the *omp25* gene (a Δ*omp25* mutant) was created.[a] The resulting isolate was termed BA25 for *B abortus* Δ*omp25* mutant. Analysis of this mutant in the murine BALB/c brucellosis model indicated that BA25 was attenuated.[a] At 48 hours after infection, a significant decrease was observed in the ability of BA25 to replicate inside cultured murine macrophages, compared with the virulent parental strain. Inoculation of mice with BA25 resulted in mean **colony-forming units (CFU)** per spleen counts that were at the 2.1 log limit of detection by 18 weeks after infection. In comparison, mice infected with the parental strain, *B abortus* 2308, had > 2 logs more bacteria at the same time point. Complementation of BA25 with the broad-host-range plasmid pBBR1MCS4 containing the complete *omp25* gene restored the CFU per spleen counts to that of strain 2308-infected mice.[a]

On the basis of these studies in mice with the *B abortus* Δ*omp25* mutant, it was hypothesized that the lack of the Omp25 protein would affect the virulence of BA25 in the cattle. To determine whether the *B abortus* mutant was attenuated, in vitro and in vivo experiments were instituted with BA25 in cattle. In vitro, the ability of the mutant to survive and replicate in bovine professional phagocytes and chorionic trophoblasts was assessed. Cattle in late gestation were also infected with BA25 to ascertain the potential of the mutant to colonize and cause abortions in cattle. Therefore, the purpose of the study reported here was to determine the virulence of BA25 in cattle.

Materials and Methods

Bacterial strains—*Brucella abortus* strain 2308 is a virulent challenge strain.[1,17,18] The creation and initial characterization of the *B abortus* Δ*omp25* mutant BA25 from strain 2308 has been described.[a] Brucellae were grown on **Schaedler blood agar plates (SBA)**[b] at 37 C in a 5% CO_2 atmosphere. The BA25 mutant was grown on SBA supplemented with kanamycin (45 µg/ml).[c] Inoculation doses of each strain were prepared as described[19] and snap frozen in liquid nitrogen prior to storage at –80 C. Immediately prior to animal inoculation, samples were thawed and diluted in sterile saline (0.9% NaCl) solution to the appropriate concentration. All inoculums were verified as correct on the day of use by serial dilution and plating on the appropriate media.

Bovine neutrophil killing assay—The bovine neutrophil killing assay was performed as described.[20] Briefly, bovine peripheral blood was obtained from a donor with no history of brucellosis vaccination with either strain 19 or RB51 and was negative on the Card test for agglutinating antibodies against *B abortus*.[21] An enriched neutrophil population was obtained from whole blood samples, using centrifugation and RBC lysis with sterile distilled water. Ninety-six well plates were seeded with 2×10^5 cells/well. The resistance of opsonized *B abortus* strain 2308 and BA25 to killing by cultured bovine neutrophils was determined by incubating each strain with these cells. At 0, 10, 30, 60, and 120 minutes after infection, neutrophils were lysed with 0.1% deoxycholate,[c] and viable counts of bacteria acquired by serial dilution and plating on SBA. Four replicate wells for each strain were evaluated at each time point, and the experiment was repeated 3 times. The percentage survival for each strain was determined by dividing the number of bacteria present at a particular sampling time by the number of brucellae added to the neutrophils at time zero and multiplying by 100.

Bovine macrophage killing assay—The bovine macrophage-killing assay was also performed as described.[20] The same donor used to obtain neutrophils was also used to acquire bovine macrophages. Phagocytes were obtained from whole blood, using Histopaque 1077.[c] Ninety-six well plates were inoculated with 5×10^5 cells/well and cells were incubated for 72 hours at 37 C in a humidified atmosphere containing 5% CO_2. After 72 hours of culture, macrophages were washed and inoculated with 5×10^7 CFU/well of opsonized BA25 or strain 2308. Following a 2-hour period of phagocytosis at 37 C, all extracellular bacteria were killed by the addition of gentamicin (50 µg/ml)[c] for 1 hour at 37 C. Following this 1-hour treatment, the high-concentration gentamicin was removed and replaced with media containing gentamicin at a low concentration (12.5 µg/ml). At 0, 24, and 48 hours following high concentration gentamicin treatment, macrophages were washed, and cells were lysed with 0.1% deoxycholate. The number of viable intracellular brucellae was determined by serial dilution and plating on the appropriate media. Percentage survival was calculated as previously described. Time zero was defined as the point when the high-concentration gentamicin was removed from the cells. As with the neutrophils, 4 replicate wells for each strain were evaluated at each time point, and the experiment was repeated 3 times.

Bovine trophoblast cell lines—Three bovine trophoblast cell lines of different gestational age were obtained for this study. An early-gestational trophoblast cell line[d] that was established from a 13- to 15-day-old bovine embryo[22] as well as a mid-gestational cell line[e] derived from the placentomal tissue of a bovine placenta in the fifth month of gestation were provided for use in this study.[23] A late-gestation trophoblast cell line[f] established from the placenta of a bovine dam in the eighth month of gestation[7] was also employed.

All 3 cell lines were maintained in a complete medium consisting of a 1:1 mixture of Ham's F12 nutrient medium[c] and **Dulbecco's modified Eagle medium**[c] **(DMEM**; pH 7.3). The media was supplemented with selenium (5 ng/ml), transferrin (5 µg/ml), insulin (5 µg/ml), epidermal growth factor[c] (10 ng/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% **fetal calf serum (FCS)**.[g] All cells were grown in 25 cm^2 flasks at 37 C in a humidified atmosphere containing 5% CO_2.

Bovine trophoblast infection assay—Prior to infection, each cell line was passaged from the 25-cm^2 flasks into 24-well plates at a concentration of 1×10^5 cells/well with 1 ml of antibiotic-free media per well. Twenty-four hours following cell passage, inoculation doses of *B abortus* strain 2308 and the Δ*omp25* mutant BA25 were thawed and diluted in warm antibiotic-free cell media. Either bacterial strain was added to the trophoblasts at 1×10^7 CFU/well, resulting in a 1:100 ratio of cells to bacteria. Bacteria were incubated with the trophoblasts for 10 hours at 37 C with 5% CO_2.

At 10 hours after infection, media was removed, and all extracellular bacteria were killed by incubation at 37 C with high-concentration gentamicin (50 µg/ml) diluted in warm

DMEM or RPMI media.[c] One hour later, the high-concentration gentamicin media was removed and replaced with the complete culture media containing a low concentration of gentamicin (12.5 µg/ml). At 0, 12, 24, 36, and 48 hours following removal of the high-concentration gentamicin, cells were gently washed with 1 ml of warm PBS solution containing 1% FCS. Following 1 wash, 800 µl of PBS solution was added to each well, and the entire plate was frozen at –80 C for later bacteriologic analysis. All 3 cell lines were inoculated with both bacterial strains with 3 replicates/time point. The entire experiment was repeated 3 times.

Following completion of each experiment, the 24-well plate was thawed at 37 C, and 0.1% deoxycholate was added to each well to lyse the cells. Following a 5-minute incubation at room temperature (20 C), the wells were serially diluted in PBS solution and plated on SBA to obtain the viable CFU per milliliter. Percentage survival was calculated by dividing the number of bacteria present at a particular sampling time by the number of brucellae added to the trophoblasts at time zero and multiplying by 100. Time zero was defined as the point when the high-concentration gentamicin was removed from the cells.

Cattle—Twenty pregnant mixed-breed cows were obtained from private herds. For all cows, results of a standard Card test were negative for *Brucella*-specific antibodies, and there was no history of strain 19 or RB51 vaccination. Serologic analysis by western immunoblot, using smooth and rough *B abortus* cell lysates, failed to reveal evidence of previous vaccination.[24] Throughout the course of the study, all cattle were housed in a USDA Animal and Plant Health Inspection Service-approved, restricted-access, large animal isolation facility located at the Louisiana State University AgCenter, Baton Rouge, Louisiana. At the conclusion of the study, the adult cows were euthanatized at a government-inspected abattoir by use of the captive bolt and exsanguination method. Live calves were euthanatized at the research facility and disposed of by incineration.

Bovine study—At approximately 220 days gestation, each cow was inoculated with 1×10^7 CFU of either *B abortus* strain 2308 or BA25 by introducing 50 µl of the inoculum into the conjunctival sac of each eye. Ten cows were inoculated with the virulent parental strain, whereas an additional 10 were inoculated with the Δ*omp25* mutant. Following inoculation, cows were monitored daily for parturition.

The birth status of the calf was recorded as either healthy, weak, or aborted. Within 12 hours of parturition, the calf was euthanatized, if alive, and a necropsy was performed. Samples obtained from the calf for bacteriologic analysis included the abomasal fluid and lung. Tissue samples were frozen at –4 C for later analysis, and the abomasal fluid was swabbed the day of collection on Farrell's selective medium.[25] Two days following birth, milk samples from all 4 quarters of the mammary gland and intrauterine fluid samples were obtained from the dam. Both fluid samples were swabbed the day of collection on Farrell's selective medium. If *B abortus* was not cultured from either the milk or uterine fluid, a second sample was obtained 2 weeks later for additional bacteriologic analysis. At approximately 35 days after parturition, dams were necropsied, and tissue samples were obtained from the supramammary lymph node, spleen, and liver for the culture of brucellae. Blood was also obtained prior to infection, following parturition, and at necropsy for serologic analysis.

Bacteriologic analysis—All tissue samples were thawed and homogenized in a sterile saline solution and plated onto Farrell's selective medium containing 5% bovine blood; the limit of detection with this system is 13 CFU/g or ml. Plates were incubated for 14 days at 37 C in a 5% CO_2 atmosphere. *Brucella abortus* was identified on the basis of urease and oxidase reactions, colony morphologic characteristics, growth rate, smooth phenotype by acriflavine, and Gram-stain reaction.[21] Strain BA25 was differentiated from *B abortus* strain 2308 by being kanamycin-resistance at 45 µg/ml.

Serologic analysis—Sera were analyzed by use of the Card test[21] and western immunoblot.[26] The Card test, an accepted field agglutination test, was performed as described.[21] Western immunoblot analysis was performed, using cell lysates from the smooth *B abortus* strain 2308 and rough *B abortus* strain RB51. Cell lysates were prepared by sonication and dilution in Laemmli sample buffer as described.[24] Electrophoresis and western immunoblot were performed as described.[26]

Statistical analyses—For neutrophil, macrophage, and trophoblast assays, statistical comparisons between experimental groups were performed by use of the Student *t*-test for parametric data and the Mann-Whitney ranked sum test for nonparametric data.[27] The rate of abortion between cattle inoculated with strain 2308 or BA25 was compared, using the Fisher exact probability test, whereas median CFU per gram of tissue was compared by use of the Mann-Whitney ranked sum test.[27] Values of $P < 0.05$ were considered significant for all 3 tests. All statistics were performed with statistical software.[h]

Results

Bovine neutrophil killing assay—To determine whether the lack of the Omp25 protein would render BA25 more susceptible to killing by professional phagocytes, the Δ*omp25* mutant and virulent parental strain were exposed to cultured bovine neutrophils. The difference in percentage survival between BA25 and strain 2308 was not significant at 10, 30, 60, or 120 minutes after infection. At 30 minutes after infection, the percentage survival for both strains decreased to approximately 30% and remained the same thereafter (data not shown). The experiment was repeated 3 times with no significant deviations observed between trials.

Bovine macrophage killing assay—To ascertain whether the Δ*omp25* mutant would be more susceptible to the bactericidal mechanisms of the macrophage, a series of in vitro killing assays were performed with freshly isolated bovine macrophages (**Fig 1**). By 24 hours after infection, the parental strain had decreased in viable numbers by 28%, whereas BA25 had decreased 93% ($P < 0.001$; n = 4). At 48 hours after infection, strain 2308 replicated to 549%, whereas the mutant recovered to only 33% ($P < 0.01$; n = 4). The experiment was repeated 3 times; while individual variances were observed, BA25 was consistently killed at a higher rate than *B abortus* strain 2308.

Bovine trophoblasts infection assay—The *B abortus* Δ*omp25* mutant was examined by tissue cultural technique in late-, mid-, and early-gestational bovine trophoblasts to determine whether the lack of Omp25 rendered BA25 attenuated in these chorionic cells. In the late-gestational cell line, the Δ*omp25* mutant replicated at lower numbers than the parental strain 24, 36, and 48 hours following infection (**Fig 2**). By 24 hours after infection, strain 2308 replicated to 469% survival, whereas BA25 recovered to 85% sur-

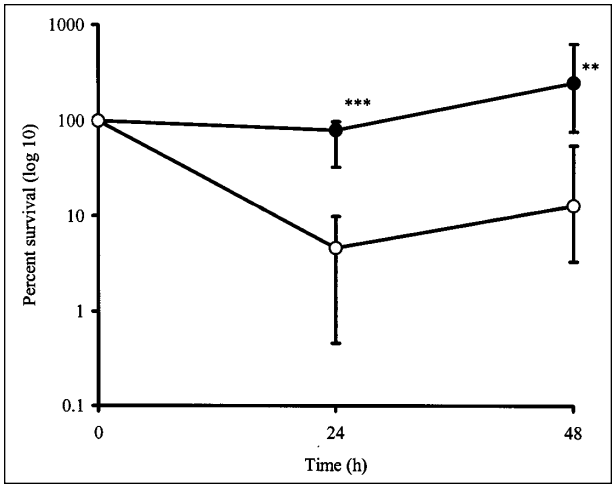

Figure 1—Replication of *B abortus* strains 2308 and BA25 in late-gestational bovine chorionic trophoblasts. Data presented are the results of 1 representative experiment with 3 repetitions at each time point. Vertical bars indicate SEM. Closed circles = Strain 2308. Open circles = Strain BA25. Significant (**$P < 0.01$, ***$P < 0.001$) differences detected (on the basis of results of the *t*-test; n = 3).

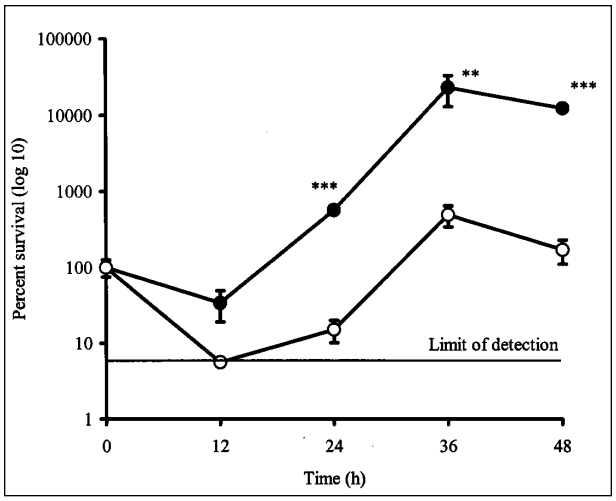

Figure 2—Killing of *Brucella abortus* strains 2308 and BA25 opsonized with hyperimmune murine serum by cultured bovine macrophages. Data presented are the results of 1 representative experiment with 4 repetitions at each time point. Vertical bars indicate the 25% and 75% quartiles. Closed circles = *B abortus* strain 2308 (median). Open circles = *B abortus* mutant strain BA25 (median). Significant (**$P < 0.01$, ***$P < 0.001$) differences detected (on the basis of results of the Mann-Whitney rank sum test of nonparametric data; n = 4).

vival ($P < 0.001$; n = 3). Significant differences in percentage survival between the 2 strains were also observed at 36 ($P < 0.01$; n = 3) and 48 ($P < 0.001$; 3) hours after infection. Although BA25 did not replicate to the same extent as strain 2308, the mutant was able to surpass the inoculum at 36 and 48 hours after infection. Two additional replicates of this assay produced no significant variations. In the mid- and early-gestational trophoblast cell lines, a significant decrease in the percentage survival of BA25 was also observed, compared with strain 2308 (n = 3; data not shown).

Cattle study—To assess the pathogenicity of BA25, pregnant cattle in late gestation were exposed to either the Δ*omp25* mutant or the virulent parental strain, *B abortus* 2308. Inoculation of 10 pregnant cows with virulent strain 2308 resulted in 5 of the 10 dams aborting (**Table 1**). In contrast, only 1 of 10 cows inoculated with BA25 aborted. The virulent parental strain was isolated by bacteriologic cultural technique from every dam and calf inoculated with strain 2308. The Δ*omp25* mutant was recovered from 5 of 10 dams and 3 of 10 calves. Four of 10 dams exposed to BA25 did not develop antibodies against *B abortus* nor were they colonized by the mutant. All of the cows inoculated with strain 2308 developed antibodies against *B abortus* and were colonized with this strain.

Table 1—Abortion rate, colonization rate, and serologic response of 20 pregnant cows inoculated with either *Brucella abortus* strain 2308 or BA25

Observation	BA25 group (n = 10)	Strain 2308 group (n = 10)
Abortion rate	10*	50
Calf: colonization		
Abomasal fluid	20***	100
Fetal lung	30*	90
Cow: colonization		
Uterine fluid	30**	100
Milk	50*	100
Supramammary LN	40* (3/7)†	90 (9/10)
Liver/spleen	0	0
Serologic analysis‡		
Positive response	60	100

All data are presented as percentage of cows with positive test results out of 10 cows/treatment group. Liver and spleen tissue were homogenized and cultured together.

LN = Lymph node.

†Only 7 tissue samples were cultured. ‡Detection of antibodies specific for the O-side chain by use of western immunoblot analysis.

Significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) by use of the Fischer exact probability test; n = 10.

The median CFU per gram of tissue obtained from the 2 experimental groups was compared by use of a Mann-Whitney ranked sum test. The 3 supramammary lymph nodes colonized by BA25 had a significantly lower median CFU per gram of tissue than the strain 2308-infected cows. The median CFU per gram of tissue for the dams inoculated with 2308 was 1,000 CFU, with 25% and 75% quartiles of 192 and 3,250 CFU, respectively. In contrast, the 3 colonized tissue samples from the BA25-infected dams had a median of 60 CFU/g, with 23 and 128 CFU for quartiles.

Discussion

On the basis of the in vitro analysis in bovine phagocytes and chorionic trophoblasts, BA25 may be unable to adapt to the intracellular environment as quickly as virulent *B abortus* strain 2308. Incubation of BA25 with bovine macrophages resulted in a significant ($P < 0.01$) decrease in percentage survival at 48 hours after infection (n = 4). The data from bovine macrophages indicated that the lack of Omp25 may render BA25 more susceptible to the bactericidal environment of the activated macrophage.

In bovine trophoblasts from late-, mid-, and early-gestation, the Δ*omp25* mutant was again unable to replicate inside the cell to the same extent as the virulent parental strain, *B abortus* 2308. In all 3 cell lines, both strains decreased by more than half a log at 12 hours

after infection. This may reflect a period of adjustment where the bacterium modulates the expression of different genes involved in intracellular growth. Although trophoblasts do not have the antimicrobial activity of macrophages, the organism must still adapt to this intracellular environment. Once inside the trophoblast, *B abortus* will eventually metabolize the sugar erythritol, preferentially over other carbon sources, which is implicated in the tropism of brucellae to the uterus.[6] The Δ*omp25* mutant can metabolize erythritol in vitro (data not shown). The inability of BA25 to adjust to the intracellular environment of the trophoblast is reflected in the lower percentage survival of the Δ*omp25* mutant at 36 and 48 hours after infection.

The delayed growth of BA25 in macrophages and trophoblasts may explain the attenuation of this mutant in vivo. If the Δ*omp25* mutant is unable to survive and replicate inside bovine macrophages, the organism may be eliminated from the draining lymph node, preventing dissemination and eventual colonization of the developing fetus. Furthermore, the inability of BA25 to replicate inside the late-gestational bovine trophoblasts to the same extent as strain 2308 may also have important consequences in the late-term abortion process. If the bacteria cannot replicate efficiently in the chorionic membranes, the chance of fetal infection and subsequent abortion is lowered.

In the cattle of our study, the *B abortus* Δ*omp25* mutant was attenuated, compared with the virulent parental strain. Of the 10 dams exposed to BA25, 4 did not develop *Brucella* antibodies nor were they colonized. These cows may have eliminated the mutant in the draining lymph node, thereby preventing spread throughout the host. One dam did develop antibodies against *B abortus*, but culture results for brucellae were negative in both the dam and calf. This dam was colonized for a sufficient time to develop an antibody response but was able to eliminate the mutant prior to parturition. The inability of BA25 to replicate to the same extent as strain 2308 in bovine macrophages during the early stages of infection may have resulted in 5 of 10 pregnant cattle eliminating the mutant prior to parturition.

The remaining 5 pregnant dams inoculated with the Δ*omp25* mutant were colonized by BA25, but only 3 had calves that had positive test results for *Brucella* organisms. The fact that only 3 of 5 colonized dams had calves that were likewise positive for *B abortus* may reflect the inability of BA25 to replicate efficiently inside the late-gestational trophoblast. Following enhanced killing of the mutant by activated macrophages in the draining lymph node, lower numbers of the Δ*omp25* mutant were disseminated throughout the host. Those bacteria that did succeed in colonizing the chorionic membrane were unable to replicate as efficiently as the virulent parental strain. As a result, the necrosis to fetal membranes was decreased, and only 3 of 5 calves were colonized in utero.

The lack of the Omp25 protein did render BA25 attenuated in the cattle of this study. The role of Omp25 in the replication of *B abortus* in bovine macrophages and trophoblasts needs to be furthered explored. A detailed understanding of the physiologic role of this protein would provide additional information on the pathogenesis of *Brucella* spp. By understanding the role of Omp25 and other proteins in *Brucella* spp, the development of new vaccines lacking key virulence factors is possible.

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[a]Edmonds MD. *Creation and characterization of 25 kd outer membrane protein (Omp25) deletion mutants in Brucella species*. PhD dissertation, Department of Veterinary Microbiology and Parisitology, Louisiana State University, Baton Rouge, La, 2000.

[b]Difco Laboratories, Detroit, Mich.

[c]Sigma Chemical Co, St Louis, Mo.

[d]Courtesy of Stringfellow D, Auburn College of Veterinary Medicine, Auburn, Ala.

[e]Courtesy of Munson L, University of California, Davis, Calif.

[f]Courtesy of Enright F, Louisiana State University AgCenter, Baton Rouge, La.

[g]Hyclone, Logan, Utah.

[h]Sigma Stat Statistical Software, version 1.0, Jandel Scientific, San Rafael, Calif.

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