Immune Responses and Protection against Experimental Brucella suis Biovar 1 Challenge in Nonvaccinated or B. abortus Strain RB51-Vaccinated Cattle

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Received 9 August 2010/Returned for modification 9 September 2010/Accepted 1 October 2010

Twenty Hereford heifers approximately 9 months of age were vaccinated with saline (control) or 2 \(\times 10^{10}\) CFU of the Brucella abortus strain RB51 (RB51) vaccine. Immunologic responses after inoculation demonstrated significantly greater (\(P < 0.05\)) antibody and proliferative responses to RB51 antigens in cattle vaccinated with RB51 than in the controls. Pregnant cattle received a conjunctival challenge at approximately 6 months of gestation with 10\(^7\) CFU of B. suis bv. 1 strains isolated from naturally infected cattle. The fluorescence polarization assay and the buffered acid plate agglutination test had the highest sensitivities in detecting B. suis-infected cattle between 2 and 12 weeks after experimental infection. Serologic responses and lymphocyte proliferative responses to B. suis antigens did not differ between control and RB51 vaccinees after experimental infection. No abortions occurred in cattle in either treatment group after challenge, although there appeared to be an increased incidence of retained placenta after parturition in both the control and the RB51 vaccination treatment groups. Our data suggest that the mammary gland is a preferred site for B. suis localization in cattle. Vaccination with RB51 did not reduce B. suis infection rates in maternal or fetal tissues. In conclusion, although B. suis is unlikely to cause abortions and fetal losses in cattle, our data suggest that RB51 vaccination will not protect cattle against B. suis infection after exposure.

The United States declared that all cattle in all states are free from Brucella abortus infections in 2009. The elimination of B. abortus from cattle was the result of national eradication activities that began in 1934, with total costs exceeding $10 million (18). Although several wildlife reservoirs remain a threat for reintroduction, ongoing monitoring activities are designed to detect any events resulting in transmission to domestic cattle (11).

Although B. abortus has been eliminated from domestic cattle, the prevalence of B. suis in feral swine has emerged as a significant problem for domestic cattle. Feral swine populations continue to increase in the United States, and illegal transportation continues to expand their range into new states or regions (9). Contact with infected feral swine has led to B. suis infections in a large number of cattle, particularly in the south and southeastern United States. Cattle infected with B. suis test seropositive on brucellosis surveillance tests, and the antibody responses cannot be readily differentiated from those due to infection with B. abortus. At this time, data on the temporal characterization of the serologic responses of cattle after acute infection with B. suis are lacking.

In many states, calfhood vaccination with B. abortus strain RB51 (RB51) is still utilized in domestic cattle. However, the efficacy of RB51 vaccination in protecting cattle against B. suis infection is unknown. In this study we characterized the serologic responses of cattle to B. suis infection and compared the pathogenesis and tissue localization of B. suis in pregnant RB51-vaccinated and control cattle after experimental challenge.

MATERIALS AND METHODS

Vaccine and challenge cultures. A master seed stock of RB51 was obtained from G. Schurig (Virginia Polytechnic Institute and State University, Blacksburg, VA) and, after one passage on tryptose agar (Difco Laboratories, Detroit, MI), was designated the ARS/1 seed stock of RB51. For experimental use, RB51 bacteria from the ARS/1 seed stock were grown on tryptose agar for 48 h at 37°C. For the enzyme-linked immunosorbent assay (ELISA), RB51 suspensions (1.3 \(\times 10^{12}\) CFU/ml) were inactivated by \(\gamma\)-irradiation (1.4 \(\times 10^{10}\) rads). Following irradiation, suspensions were washed in 0.15 M sodium chloride (saline) and stored in 1-ml aliquots at \(-70\)°C.

For serologic and proliferation assays after challenge, the smooth strain B. suis 1330 (which expresses the O side chain of the lipopolysaccharide) and the rough strain B. suis 353-1 (which does not express the O side chain of the lipopolysaccharide) were grown on tryptose agar for 48 h at 37°C in 5% CO\(_2\). Bacteria were washed from the agar using sterile saline, adjusted to approximately 10\(^{10}\) CFU/ml, and killed by addition of methanol (60:40 [vol/vol] methanol-bacteria solution) and incubation at 4°C for 5 days. Bacteria were washed once in saline and stored at \(-70\)°C until use.

To ensure that the B. suis strains used for experimental challenge were infectious to cattle, four B. suis field strains isolated from Texas cattle were obtained from the National Veterinary Service Laboratory (USDA, APHIS, NVSL). These strains were individually grown on tryptose agar for 48 h at 37°C in 5% CO\(_2\). Bacteria were washed from agar plates using sterile saline, and strains were individually adjusted to approximately 10\(^{10}\) CFU using a spectrophotometer. Equal volumes of all strains were then combined and mixed to prepare the challenge inoculum. The concentration of B. suis in the challenge inoculum was determined by dilution of the suspension in saline and standard plate counts.

Vaccination. Twenty Hereford heifers approximately 9 months of age were obtained from a brucellosis-free herd. One-half of the heifers (n = 10) were randomly assigned to receive 10\(^{10}\) CFU of a commercial lyophilized RB51 vaccine (Colorado Serum Company, Denver, CO). The RB51 vaccine was prepared...
in accordance with the manufacturer’s instructions and subcutaneously admin-
istered in the superficial cervical region. The remaining heifers received 2 ml of
saline administered subcutaneously in a similar manner. The concentration of
RB51 in the inoculum was determined by dilution of the vaccine in saline and
standard plate counts.

**Postvaccination serologic responses.** Blood samples were collected by jugular
venipuncture prior to vaccination and at 4, 8, 12, 16, and 24, and 24 weeks postin-
oculation. Blood was allowed to clot for 12 h at 4°C and centrifuged. Serum was
divided into 1-ml aliquots, frozen, and stored at −70°C.

Titers of antibodies to *Brucella* were determined by a previously described
ELISA in which γ-irradiated RB51 is used as antigen (17).

**Postvaccination lymphocyte proliferation.** At 4, 8, 12, 16, and 24 weeks
after vaccination, blood was obtained from the jugular vein of all cattle and
placed into an acid-citrate dextrose solution. Peripheral blood mononuclear cells
(PBMCs) were enriched by density centrifugation using a Ficoll-sodium diatrizo-
ate gradient (Sigma Diagnostics, Inc., St. Louis, MO). Peripheral blood mono-
nuclear cells were diluted in RPMI 1640 medium to 1 × 10^7 viable cells per ml,
as determined by trypan blue dye exclusion.

Fifty microliters of each cell suspension, containing 5 × 10^5 cells, was added to
each of two separate flat-bottom wells of 96-well microtiter plates that con-
tained 100 μl of RPMI 1640 medium only or RPMI 1640 medium containing
γ-irradiated RB51 (10^10 to 10^11 bacteria per well). Cell cultures were incubated for
7 days at 37°C in 5% CO_2. After 7 days of incubation, cell cultures were pulsed
with 1.0 μCi of [^3]H]thymidine per well for 18 h. Cells were harvested onto glass
filter mats and counted for radioactivity in a liquid scintillation counter.

**Experimental B. suis challenge.** Bovine heifers were pasture bred beginning at
11 months after vaccination (approximately 20 months of age). Pregnancy status
and stage of gestation were determined by rectal palpation at between 40 and 90
days of gestation. Two to 3 weeks prior to challenge, pregnant cattle were trans-
ferred to a biolevel 3 containment facility, where they were housed for the
duration of the study. On the basis of palpation data, pregnant cattle were
intraconsciously challenged at between 170 and 180 days of gestation with 1 ×
10^7 CFU of field strains of *B. suis* (50 μl of inoculum per eye). Conjunctival swabs
were obtained at 3 days after challenge and cultured on tryptose agar containing
5% bovine serum.

**Postchallenge serology.** Prechallenge blood samples were obtained via the
jugular vein and at 2-week intervals thereafter. Serum was preserved as described
for postvaccination samples and stored at −70°C. Serum samples were submitted
to the National Veterinary Services Laboratory for analysis by the card test,
rivalon (RIV) test, buffered acid-plate agglutination (BAPA) test, fluorescence
polarization assay (FPA), complement fixation (CF) test, and the standard tube
agglutination (STAT) test using standard procedures (USDA, APHIS, NVSL,
2010). Results of the card and BAPA tests were considered positive when
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**Postvaccination.** Standard plate counts indicated that cattle in the RB51 treatment group were initially vaccinated with
2.0 × 10^{10} CFU.

Compared to the controls, cattle vaccinated with RB51 demon-
strated greater (*P* < 0.05) mean antibody responses to RB51
at 4, 8, 12, 16, and 20 weeks (Fig. 1). Cattle vaccinated
with RB51 had the highest titers at 4 or 8 weeks after vaccination.
In a similar manner, at 8, 12, and 16 weeks, vaccinated heifers
had greater (*P* < 0.05) mean proliferative responses to RB51
bacteria than nonvaccinated cattle (Fig. 2).

**RESULTS**

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Standard plate counts indicated that the mean challenge dose was recovered at 3 days after challenge from eye swabs of 7 of 9 control animals evaluated and 6 of 8 RB51 vaccinees. Eye swabs were not taken from 1 control animal and 1 RB51 vaccinee due to disposition issues.

Many of the brucellosis serologic tests used in this study (card, RIV, STAT, and CF tests) were very poor at detecting antibody responses to B. suis between 2 and 12 weeks after experimental infection (Table 1). The BAPA test and FPA appeared to be the most sensitive for detecting B. suis-infected animals, with positive responses peaking at between 4 and 8 weeks after experimental infection. Serologic data did not suggest statistical differences (P < 0.05) in the sensitivities of the BAPA test and FPA when the results for control and RB51-vaccinated cattle were compared.

Postchallenge. Ten controls and 9 RB51 vaccinees were found to be pregnant and used for experimental challenge. Standard plate counts indicated that the mean challenge dosage was 7.9 × 10^6 CFU. Brucella suis was recovered at 3 days after challenge from eye swabs of 7 of 9 control animals evaluated and 6 of 8 RB51 vaccinees. Eye swabs were not taken from 1 control animal and 1 RB51 vaccinee due to disposition issues.

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Representative data for proliferative responses of peripheral blood mononuclear cells to killed B. suis strains 1330 and 353-1 are presented in Fig. 2. Overall data suggested that proliferative responses to B. suis antigens did not differ (P > 0.05) between control and RB51 vaccination treatments after experimental challenge.

All control animals gave birth to full-term viable calves at between 10 and 15 weeks after challenge. With the exception of 1 RB51 vaccinee which was euthanized prior to calving, the remaining 8 animals gave birth to full-term, viable calves at between 11 and 16 weeks postchallenge. All full-term calves had crown-rump lengths of 94 to 119 cm. The RB51 vaccinee euthanized prior to parturition at 16 weeks after challenge was estimated to be in the early part of the third trimester of gestation, on the basis of a fetus crown-to-rump length of 57 cm. Retained placenta was noted for 3 of 10 cows (30%) in the control treatment group and 3 of 8 cows (38%) in the RB51 treatment group that delivered after full-term gestation.

The ability to recover B. suis from samples obtained at necropsy did not differ (P > 0.05) between control and RB51 vaccination treatments. The B. suis challenge was recovered from all nonvaccinees (10/10) and 75% (6/8) of RB51 vaccinees (Table 2). There was a trend for greater recovery from the mammary gland, fetal tissues, and blood, with lower levels of recovery from the uterine placentome, vaginal swabs, and maternal lymph nodes occurring. Although isolation from blood had the greatest recovery of B. suis in fetal samples (13/18), isolation from rectal swabs (12/18), gastric contents (8/18), and bronchial lymph node (7/18) was also demonstrated to have a high correlation with culture status.

**DISCUSSION**

The most important conclusion that can be made on the basis of the results of this study is that calfhood vaccination of cattle with RB51 did not protect against infection with B. suis, shedding of B. suis in the milk, or vertical transmission of B. suis to offspring. Our data correlate with data from previous reports describing a predilection for localization in the mammary gland or milk after natural infection (8). Our data are also comparable to those from a small pilot study in which......
B. abortus strain 19 vaccination failed to protect heifers from infection or shedding of B. suis in the milk after experimental intramammary challenge (19). On the basis of these data of a study looking at acute infection, RB51 vaccination is unlikely to be an effective management tool for addressing the regulatory issues associated with transmission of B. suis from feral swine to cattle.

Our data also suggest that B. suis infection is unlikely to cause abortion or weak fetuses but may increase the incidence of retained placenta in cattle. As retained placenta has been associated with B. abortus infection in cattle, a similar role for B. suis in this clinical syndrome is not surprising. It must be recognized that retained placenta in cattle has multiple etiologic mechanisms, known and unknown (2). However, in areas in which brucellosis is endemic in feral swine, our data might suggest that cows with retained placenta should be considered for further diagnostic evaluation to determine if they are infected with B. suis.

Isolation of B. suis from cattle under field conditions is not a recent phenomenon. In the early 1980s, isolation of B. suis from cattle in Australia was reported (5, 6). More recent reports from South America have also reported B. suis isolations from cattle (10).

Particularly in the southern United States, B. suis appears to be responsible for the increasing numbers of cattle testing positive on brucellosis surveillance tests in areas reported to be free of B. abortus. Determining how to manage these reactor cattle is causing difficulties for regulatory personnel and increased economic costs for producers.

Our data also support the conclusion that some brucellosis surveillance tests (card, rivanol, standard tube agglutination, and complement fixation tests) have poor sensitivities in detecting B. suis infection in cattle (7, 13). The card test is generally considered to have a high sensitivity for detection of B. abortus infection in cattle and is often used as a screening test. The rivanol and complement fixation tests are generally considered to have high specificities in detecting antibodies to B. abortus in cattle and are often used as confirmatory tests (7, 14). The inability of these four tests to detect B. suis infections in cattle could be due to use of reagents in these tests that are based on B. abortus antigens rather than B. suis antigens.

Our data do differ from data from a previous study of naturally infected cattle, in that all four tests that had low sensitivities in our study evaluated responses after acute infection, whereas the other study evaluated responses after chronic infection.

In comparison, FPA and the BAPA test had good sensitivities in detecting bovine antibodies against B. suis in our study. It should be noted that the sensitivities of FPA and the BAPA test appeared to peak at between 4 and 8 weeks after infection. These tests are often used as confirmatory tests in cattle due to their high specificities in detecting antibodies against B. abortus (7, 12). The diagnostic antigen used for the FPA is O-polysaccharide extracted from B. abortus cells, whereas the BAPA test antigen is made using whole B. abortus bacteria. Both B. suis

**TABLE 2. Recovery of B. suis from maternal or fetal tissues obtained at necropsy after conjunctival challenge from nonvaccinated cattle and cattle receiving 10^10 CFU of B. abortus strain RB51 as a calfhood vaccine**

| Infection or sample | No. of cattle responding/no. of cattle tested |
|---------------------|---------------------------------------------|
|                     | Control                                      | Vaccines                                  |
| Mammary infection   | 7/10                                        | 5/8                                       |
| Uterine infection   | 3/10                                        | 2/8                                       |
| Maternal lymph nodes| 3/10                                        | 3/8                                       |
| Maternal infection  | 10/10                                       | 6/8                                       |
| Fetal infection     | 8/10                                        | 6/8                                       |
| Maternal blood      | 5/10                                        | 4/8                                       |
| Fetal blood         | 7/10                                        | 6/8                                       |
| Vaginal swab        | 1/10                                        | 2/8                                       |
| Conjunctival swab   | 2/10                                        | 1/8                                       |

*a* Mammary infection is defined as recovery from milk, mammary gland, or supramammary lymph node.

*b* Uterine infection is defined as recovery from placenta, vaginal swab, or internal iliac lymph node.

*c* Maternal infection is defined as recovery from any maternal tissue or swab.

*d* Fetal infection is defined as recovery from any fetal tissue or swab.

![FIG. 3. Proliferative responses to 10^8 CFU of methanol-killed B. suis rough strain 353-1 (A) or smooth strain 1330 (B) by peripheral blood mononuclear cells of control and RB51-vaccinated cattle at 10 weeks (n = 7 and 8, respectively) or 12 to 14 weeks (n = 4 and 5, respectively) after experimental challenge with 10^7 CFU of B. suis bv.

1. Cells were incubated at 37°C in 5% CO₂ for 7 days and pulsed for 18 h with [3H]thymidine. Results are expressed as mean stimulation indices ± SEMs. Means within a sampling time with different letters above the bars are significantly different (P < 0.05).](http://cvi.asm.org/Downloaded from)
and B. abortus are reported to predominantly have the A type of antigenic determinant on the smooth lipopolysaccharide.

Our data have some similarity to data from a small pilot study in which naive cattle were conjunctivally infected with 2.7 × 10^7 CFU of B. suis bv. 1 at between 11 and 33 weeks of pregnancy (15). Although calves from 2 of 6 pregnancies were stillborn, they were not considered abortions, as B. suis was not isolated from dams or calves. As our study used B. suis field strains isolated from cattle, this may explain the differences between the two studies in the recovery of B. suis after parturition. There were also differences in serologic responses between the studies, as the other study reported that all cattle had positive responses on the card test at 2 weeks and had STAT test titers that fell within our suspect range for up to 11 weeks. As with our study, the CF test appeared to have a low sensitivity, as only 2 of 6 cows had positive responses on this test.

In conclusion, our study suggests that RB51 vaccination of cattle will not protect against infection after exposure to B. suis bv. 1. Although it is unlikely that economic losses due to abortion will occur, cattle infected with B. suis appear to be more likely to have an increased incidence of retained placentas. Due to the increasing population of feral swine in the United States and the fact that populations in some areas are known to have a high prevalence of B. suis infection, the risk of transmission to cattle is unlikely to diminish. Reports of cattle that test seropositive for brucellosis and that are later found to be infected with B. suis are increasing. The cases of B. suis in cattle are not only causing diagnostic difficulties but are also causing concerns for regulatory personnel who are monitoring cattle populations to ensure that re-introduction of B. abortus does not occur. Due to the regulatory difficulties and economic costs associated with dealing with brucellosis-seropositive cattle of unknown etiology, there is a need for development of new diagnostics or vaccines that will assist in resolution of B. suis infections in cattle.

ACKNOWLEDGMENTS

We thank Aileen Bryant, Deb Buffington, Diane Davis, Darl Pringle, Dennis Johannes, Doug Ewing, and Katrina Bunte for their technical assistance. We also thank Beth Harris and Christine Quance of the National Veterinary Service Laboratory, Mycobacteria/Brucella section within the Diagnostic Bacteriology Laboratory, for supplying the Brucella suis strains used in the project.

The use of product names is necessary to report factually on the exclusion of the product to the exclusion of other vaccines that may also be suitable.

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