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AN INDIRECT ELISA TO DETECT THE SEROLOGIC RESPONSE OF ELK (*CERVUS ELAPHUS NELSONI*) INOCULATED WITH *BRUCELLA ABORTUS* STRAIN RB51

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ABSTRACT: An indirect enzyme-linked immunosorbent assay (ELISA) was developed to identify elk (*Cervus elaphus nelsoni*) with *Brucella abortus* strain RB51 (RB51)-specific antibodies using a mouse monoclonal antibody specific for bovine IgG_1. This test was relatively easy to perform, accurate, and easily reproducible; therefore it could be standardized for use between laboratories. In addition, we attempted to compensate for inherent variabilities encountered when comparing ELISA readings from multiple samples taken from many animals over time. Optical density (OD) readings for each sample were converted into a percent positivity value for analysis. A negative cutoff value was determined above which a sample was considered to have a significantly elevated anti-RB51 antibody level. Pre- and postvaccination sera from 64 6–8 mo old elk, divided into four groups (females subcutaneously inoculated with saline (control animals), females ballistically inoculated with RB51, females subcutaneously inoculated with RB51, and males subcutaneously inoculated with RB51) were used. All serum samples were collected between 27 April and 15 November 1995. Values for all saline controls were appropriately below the negative cutoff value. All subcutaneously and ballistically inoculated elk were serologically positive to RB51 for at least two sampling periods during the study. The difference in percent positivity values for the ballistically compared to the subcutaneously inoculated groups was not statistically significant at 8, 10, 14, or 18 wk postvaccination. This suggests that processing RB51 into lactose based pellets and ballistically inoculating elk with these pellets does not alter the detectable elk antibody response. Also, inoculated and control animals can be accurately identified with ELISA at 4–8 weeks postvaccination.

Key words: Ballistic inoculation, *Brucella abortus, Cervus elaphus nelsoni,* ELISA, elk, serology, strain RB51.

INTRODUCTION

Brucella abortus is an aerobic, facultative intracellular, Gram negative bacteria that causes disease in humans, domestic animals, and wildlife. In cattle, *B. abortus* infection may result in late term abortions, births of nonviable calves, retained placentas, hygromas, and sterility (Cunningham, 1977). Currently, the US is attempting to eradicate bovine brucellosis through a combination of quarantine procedures, serologic tests, slaughter programs, and vaccination programs as directed by the Cooperative State-Federal Brucellosis Eradication Program (United States Department of Agriculture, 1998). While the US has essentially eliminated brucellosis from the livestock industry, many other countries report a high incidence of the disease in both animal and human populations (Boschiroli et al., 2001).

In the US, *B. abortus* has been isolated from wildlife (Thorne et al., 1978; Tessaro, 1989) including bison (*Bison bison*) and elk. Elk have the potential to transmit the infection to cattle (Thorne et al., 1980). Therefore, control or elimination of *B. abortus* infection in wildlife has become a high priority. One potential control measure involves vaccinating elk with *B. abortus* RB51 (RB51), a stable, rough mutant of *B. abortus* strain 2308. Strain RB51 induces protection against challenge with

smooth virulent *Brucella* spp. in cattle (Cheville et al., 1993), swine (Edmonds et al., 2001), and mice (Jimenez de Bagues et al., 1994). A major advantage of RB51 vaccination, regardless of the frequency, dose, or route of inoculation, is its inability to induce anti-LPS associated O-side chain antibodies whose identification is the primary means of diagnosing field strain infections of *Brucella* spp (Stevens et al., 1995). Protection against *Brucella* infection in cattle is believed to rely mainly on cell mediated immunity; therefore antibody levels are not a predictor of immunity, but an indicator of exposure. A similar situation is believed to be true in bison and elk (Nicoletti and Winter, 1990).

This research was conducted to study the serologic response of elk to vaccination with RB51 and to develop an enzyme-linked immunosorbent assay (ELISA) to identify RB51 vaccinated elk. Both the subcutaneous and ballistic vaccination routes were evaluated because the ballistic route may be a practical means of vaccinating wildlife (Herriges et al., 1989).

MATERIALS AND METHODS

Animals

Sixty-four 6–8 mo old elk were captured from the National Elk Refuge in Jackson Hole, Wyoming (Teton County, Wyoming, USA: 43°30′N, 110°45′W) and housed at the Sybille Wildlife Research and Conservation Education Unit (Wyoming Game and Fish Department, Wheatland, Wyoming). These elk are the same elk studied in the published studies by Cook et al. (2002) and Gall et al. (2001). Elk were held in six pens. Both female and male elk were examined to eliminate gender bias. Saline inoculated elk served as a control group. The saline controls were commingled with the RB51 vaccinated animals therefore serving as sentinels and donors of negative control sera for the ELISA. All animals were fed high quality alfalfa hay and pellets. All elk were shown to be free of *Brucella* infections by five consecutive negative reactions in the buffered *Brucella* antigen (BBA), the standard plate agglutination (SPA), rivanol (Riv), and the complement fixation tests (CFT) (Alton et al., 1988). The elk were divided into four groups based on gender and route of inoculation of viable RB51 organisms. Female, saline control animals received no RB51 organisms. Female, ballistically inoculated animals received between 2×10^7 and 3×10^8 organisms. Both female and male, subcutaneously inoculated animals received 1×10^9 organisms. The number of RB51 organisms administered to each group was retrospectively determined through serial dilutions of each inoculum plated onto trypticase soy agar (Difco Laboratories, Detroit, Michigan, USA).

Inocula were obtained from the National Veterinary Services Laboratories (Ames, Iowa, USA) of the Animal and Plant Health Inspection Service. Ballistic inocula were prepared in biodegradable lactose-based pellets grown from the same stock of RB51 used for subcutaneous inoculation. Elk were bled 14 days prior to vaccination, at vaccination (day 0), and six times after vaccination (weeks 4, 8, 10, 14, 18, and 27 postvaccination) from 27 April to 15 November 1995.

Blood was cultured for *Brucella* at the Wyoming Game and Fish Veterinary Diagnostic Laboratory, University of Wyoming (Laramie, Wyoming) for the first 4 mo of the experiment. The hemoculture protocol followed that of Alton et al. (1988) as described in Cook et al. (2002).

Bull elk were necropsied at bimonthly intervals after inoculation. One female elk (#38) was injured during handling and was subsequently euthanized.

RB51 whole cell antigen preparation

A lyophilized stock culture of RB51 was reconstituted with sterile trypticase soy broth. Five hundred microliters of the broth solution was spread on a trypticase soy agar (TSA) plate and incubated for 48 hr at 37 C in a humidified incubator supplemented with 5% CO_2. Rough morphology of the organisms was verified by testing for agglutination in a 0.1% solution of acriflavin in distilled water and by colony uptake of 0.05% crystal violet dye. After verification, the bacteria were subcultured on 10 TSA plates and incubated for 72 hr (humidified 37 C chamber with 5% supplemental CO_2). The bacteria were harvested with 3–4 ml sterile distilled water, mixed with an equal volume of acetone, and stirred for 3 hr to kill the bacteria. The bacteria were then washed three times with sterile distilled water and pelleted by centrifugation at 6,000×G at 4 C for 10 min. Killed bacteria were resuspended in sterile distilled water to form a thick suspension and aliquoted into sterile glass vials. The vials were frozen at −70 C for 24 hr and lyophilized.

ELISA to detect anti-RB51 elk antibodies

Lyophilized RB51 was reconstituted in sterile distilled water to 5% transmittance at 525

nm to form an antigen stock solution which was stored in polypropylene tubes at 4 C for no longer than 1 wk. Immediately before use, this stock solution was centrifuged (3 min at 11,800xG), the supernatant discarded, and the original centrifuged volume of killed RB51 diluted 1:20 (v/v) in bicarbonate phosphate buffer (pH 9.6) generating the antigen working solution. Appropriate wells of 96 well medium-binding polystyrene plates (Costar Corporation, Kennebunk, Maine, USA) were coated with 200 μl of antigen working solution, the plates sealed with a parafilm sheet, and the plate incubated in a humidified chamber at 4 C overnight.

The plates were allowed to equilibrate to room temperature in a dark, humidified chamber and were washed four times with freshly prepared phosphate buffered solution containing 0.05% Tween 20 (PBST-20) using a semi-automated plate washer (NUNC Immunowash, Naperville, Illinois, USA). For each washing, wells were filled with approximately 250 μl of PBST-20, allowed to soak for approximately 20 sec, and then emptied by suction. After the fourth washing, the plates were inverted and tapped on a countertop to fully remove the washing buffer from the wells. However, the wells were never allowed to completely dry because this can cause high non-specific binding (Nielsen et al., 1996). Serum samples were diluted 1:50 in PBST-20 in a 96-well polypropylene plate immediately before dispensing into the antigen-coated wells. Many other serum dilutions were evaluated in previous trials (data not shown) which indicated that the 1:50 (v/v) serum dilution was optimal for this ELISA. Two hundred microliters of each diluted serum sample were then incubated in the RB51-coated polystyrene plates at 37 C for 30 min. All incubations in future steps were also performed at 37 C. After incubation, the wells were washed four times. Each well was then incubated with 200 μl of a 1:6,000 (v/v) dilution of mouse monoclonal anti-bovine IgG_1 (Veterinary Medical Research and Development, BIg715A, Pullman, Washington, USA) in PBST-20 for 30 min. Next, the plates were washed four times and then incubated for 30 min with 200 μl of polyclonal horseradish-peroxidase-conjugated goat IgG fraction to mouse IgG whole molecule (Cappel Laboratories, West Chester, Pennsylvania, USA) diluted 1:800 (v/v) in PBST-20. The plates were washed four times. A developing solution was prepared immediately before use by mixing 10 mg of ortho-phenylenediamine (Sigma, St. Louis, Missouri, USA) in 1 ml of 100% methanol and adding it to freshly prepared 100 ml distilled water containing 100 μl of 30% H_2O_2. The wells were incubated in the dark with 200 μl of developing solution at room temperature on a shaking platform (120 RPM) for 30 min. The developing reaction in each well was stopped with 40 μl of 0.18 M H_2SO_4. The optical density at 490 nm was determined for each well with a 96-well plate reader (Molecular Devices Corporation, Sunnyvale, California, USA).

ELISA controls

Enzyme-linked immunosorbent assays were designed to minimize the inter-well, inter-plate, inter-animal, and time variability inherent in all ELISAs. All serum sample dilutions were tested in duplicate wells to help assess inter-well variability. Positive and negative control wells were run in each plate. Wells coated with RB51 were incubated with a 1:100 dilution in PBST-20 of a bovine serum with high anti-RB51 antibody levels (Steer 66) and were designated as positive controls. The positive bovine serum was obtained from a steer immunized repeatedly with RB51 at the Virginia-Maryland Regional College of Veterinary Medicine (Blacksburg, Virginia, USA). To create identical positive control samples, a large volume of the positive bovine serum was diluted 1:100 in PBST-20, aliquoted, and frozen at −70 C. A new aliquot was thawed and used in each plate.

Three types of negative controls were run in each plate. The first, "pre-inoculation serum samples," utilized two serum samples obtained from each elk prior to inoculation with RB51 or saline. The second, "no antigen" wells, consisted of two wells for each serum sample (diluted 1:50) that were not coated with RB51 antigen stock solution. The third, "no serum" wells, consisted of two wells containing all appropriate reagents except serum. Two hundred microliters of PBST-20 were used instead of the diluted serum. Wells containing all appropriate reagents except conjugated antisera were not tested on each plate because this combination had not been shown to cause nonspecific binding in previous ELISAs (data not shown).

Calculation of percent positivity values

A percent positivity value was calculated for all samples. Percent positivity is designed to represent where a sample lies along a continuum with no binding of antibody being equivalent to 0% positivity and the antibody binding of the positive control being equivalent to 100% positivity. Therefore, the percent positivity of each sample represents the ratio of RB51 specific antibody in that sample to the amount of RB51 specific antibody in the positive control.

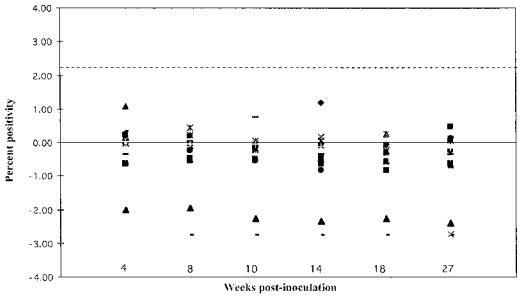

FIGURE 1. Percent positivity of serum samples (diluted 1:50) obtained from all saline inoculated elk. The horizontal dashed line corresponds to the negative cutoff value of 2.1 percent positivity.

The percent positivity of each sample was calculated with the following equation:

$$\%\,\text{positivity} = 100 \times [\text{specific OD of the test serum} \div \text{specific OD of the positive control}]$$

where:

$$\text{specific OD of test serum} = [(\text{test sample OD}) - (\text{average OD of all pre-inoculation samples from that elk})]$$

and:

$$\text{specific OD of positive control} = [(\text{positive control serum OD}) - (\text{no serum sample OD})]$$

Non-specific binding for each animal serum was partially corrected for by calculating the specific optical density for each animal's sample. Because no 'prevaccination' sample was available for the positive control sample (Steer 66), non-specific binding of the sera was approximated by subtracting the OD of a well that contained all reagents except serum from the OD of the positive control.

A negative cut-off value was determined by computing the average plus three standard deviations of the percent positivity values of all saline elk samples as described in the following equation:

$$\text{negative cutoff value} = [\text{average percent positivity value of all saline inoculated samples}] + [3(\text{standard deviation of the percent positivity of all saline inoculated samples})]$$

Any serum sample with a percent positivity value above this cutoff was considered to have a significant antibody response in this test.

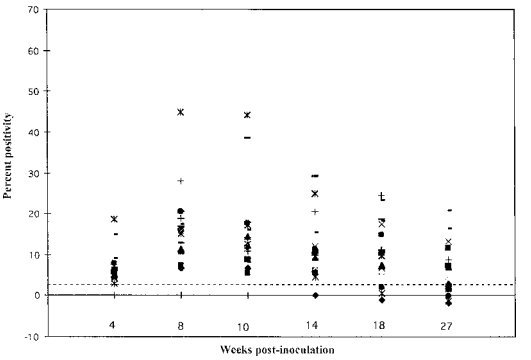

FIGURE 2. Percent positivity of serum samples (diluted 1:50) obtained from all ballistically inoculated elk. The horizontal dashed line corresponds to the negative cutoff value of 2.1 percent positivity.

RESULTS

Using the previously defined equation, a negative cutoff value of 2.1 percent positivity was calculated. Therefore, all samples with a percent positivity >2.1 were classified as having a significant anti-RB51 antibody response while samples with a percent positivity ≤ 2.1 were considered to not have a significant anti-RB51 antibody response.

All samples from each saline inoculated elk were below the negative cutoff value and samples from at least two time periods during the study for all RB51 inoculated elk were above the cutoff. Figures 1–4 are

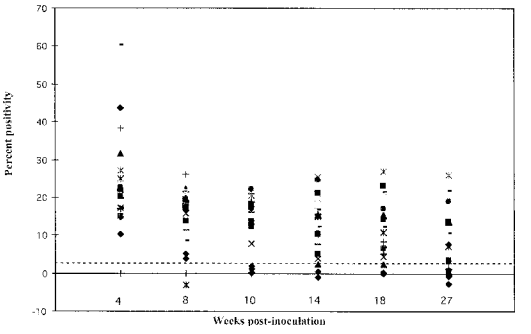

FIGURE 3. Percent positivity of serum samples (diluted 1:50) obtained from all female subcutaneously inoculated elk. The horizontal dashed line corresponds to the negative cutoff value of 2.1 percent positivity.

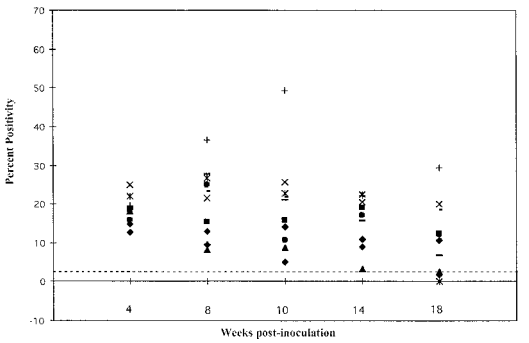

FIGURE 4. Percent positivity of serum samples (diluted 1:50) obtained from all bull subcutaneously inoculated elk. The horizontal dashed line corresponds to the negative cutoff value of 2.1 percent positivity.

graphs of the percent positivity of each elk's serum samples within their respective groups. More detailed results may be found in Colby (1997).

DISCUSSION

Mouse monoclonal bovine IgG_1 antiserum was chosen for use in the ELISA based upon Henning and Nielsen (1992) finding 100% cross-reactivity between elk and bovine IgG_1 as well as the finding that field strain *Brucella* infections cause a large IgG_1 antibody response. Because this anti-sera only identifies antibodies of the IgG_1 sub-isotype it is not a true representation of the overall antibody response. However, it does fulfill the objective of developing an ELISA to identify RB51 vaccinated elk through serology. Other anti-isotypes may be equally effective for use in the ELISA. However, anti-bovine isotypes other than anti-IgG_1 do not show 100% cross- reactivity with elk isotypes and no horseradish-peroxidase-conjugated anti-elk isotypes are currently commercially available.

Many methods for interpreting ELISA data have been described (Heck et al., 1980; Gall and Nielsen, 1994; Nielsen et al. 1996). Most methods have major limitations as they do not compensate for multi-animal, inter-well, or inter-plate variability. Also, many systems require the researcher to assign an arbitrary value above which a sample would be considered positive. This value often has no statistical validity. The analysis of data obtained with the ELISA developed in this study considered these variabilities. This ELISA is an accurate and easily reproducible method to identify elk with RB51 specific antibody titers.

Numerous controls were utilized in the ELISA. Non-specific binding of antibodies to the polystyrene plate or the RB51 antigen was evaluated in wells containing either no elk serum or no antigen coating. Duplicates of serum samples obtained prior to vaccination (14 days prior to and on the day of vaccination) were assumed to contain no specific antibodies to RB51 because the animals had not been vaccinated with any rough *Brucella* organisms and were shown to be free of exposure to smooth *Brucella* organisms. These prevaccination serum samples were used as negative controls for each animal in each plate. The magnitude and variability of the optical density value obtained with these samples was assumed to represent the normal 'background' value for the animals. Because no test had previously been developed to assess the level of anti-RB51 antibodies in elk serum, an elk serum of known antibody level was not available. As a result, bovine serum (Steer 66) with a high concentration of specific anti-RB51 antibodies was used as the positive control in each plate. In the future, hyper-immune elk sera or monoclonal anti-RB51 antibodies should be produced and used as positive control sera. The ELISA could then be standardized across multiple laboratories thereby allowing comparison of samples across laboratories.

Because the saline and the RB51 vaccinated groups belong to the same population, the saline calculated cutoff value should correctly identify $>99.5\%$ of all truly negative samples within the RB51 vaccinated groups. Using this rationale, all RB51 inoculated elk had an antibody response to vaccination with RB51. Al-

though a subsequent study has shown that the RB51 vaccine used in this study did not prevent *Brucella* infection in pregnant elk (Kreeger et al., 2000), detection of antibodies to RB51 indicates that elk were able to mount an immunologic response to the vaccine. Further study is required to determine why this immunologic response was inadequate to provide protection from infection.

The largest proportion of subcutaneously vaccinated animals can be detected at 4 wk postvaccination. All ballistically vaccinated animals were detected at 8 and 10 wk. Most animals in all vaccinated groups produced detectable anti-RB51 antibodies 27 weeks postvaccination. It may be possible to obtain significant mean percent positivity values either before 4 wk or after 27 wk postvaccination; however this cannot be determined from this study.

The average percent positivity for the ballistically inoculated group (which was vaccinated with fewer organisms than were the subcutaneously inoculated groups) was consistently lower than for the two subcutaneously inoculated groups. However when comparing values at each time for ballistically inoculated versus each subcutaneously inoculated group via a one way analysis of variance with Tukey's HSD ($P<0.05$), these differences were not statistically significant at 8, 10, 14, or 18 weeks postvaccination. This ballistic vaccination induced an antibody response similar to the one induced by subcutaneous inoculation. This finding is encouraging, because the ballistic route of inoculation may be one means of vaccinating wildlife without the complication of vaccinating non-target animals (which might occur with oral vaccinations).

Evaluating the true sensitivity and specificity of the ELISA described in this study, was not possible at the time of its development because no other method of testing elk serum for anti-RB51 antibodies had been developed. Since then, Kreeger et al., (2000, 2002) have described a modified dot blot assay designed to evaluate the antibody response of elk to RB51. While developing this ELISA, we also examined the use of dot blot assays (Colby 1997). However, in our hands, dot blot assays were labor intensive because they did not lend themselves to automation. We also found interpretation of dot blot assay results to be hard to evaluate and very subjective. Repetitions of identical dot blots resulted in variable intensity of color development and due to their variability, samples could not be compared across multiple dot blots. In our hands, using a computer image analysis program (NIH Image, US National Institutes of Health) did not resolve the evaluation problems (Colby, 1997). Alternatively, ELISAs can be easily automated and objectively evaluated. Through the use of percent positivity values and standardized reagents, they allow the comparison of samples across multiple laboratories.

Our indirect ELISA is relatively easy to perform, is suitable to automation and standardization across multiple laboratories, and correctly identifies RB51 inoculated animals. Although the current RB51 vaccine may not provide elk with adequate protection against infection with *Brucella*, multiple genetically modified RB51 organisms are currently being developed (Vemulapalli et al., 2000a, b) and will likely be evaluated for use in elk. This ELISA could be used to monitor the antibody response of elk to vaccination with genetically modified RB51 organisms as well as other rough *Brucella* organisms.

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