Journal of Wildlife Diseases, 46(1), 2010, pp. 87–94
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EVALUATION OF THE WESTERN IMMUNOBLOT AS A DETECTION METHOD FOR *BRUCELLA ABORTUS* EXPOSURE IN ELK

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ABSTRACT: *Brucella abortus* has been an important wildlife disease issue for most of the last century, especially because wildlife species are considered to be important disease reservoirs for cattle. Diagnostic uncertainty, caused in part by cross-reactions of antibodies to environmental pathogens such as *Yersinia enterocolitica* O:9 on standard *Brucella* serology, has exacerbated the challenges of managing the disease and has highlighted the need for test validation in wildlife species. The western immunoblot was evaluated for use in detecting *B. abortus* exposure in elk (*Cervus elaphus*) and for ruling out exposure to cross-reacting bacteria. Samples collected from 2003 to 2006, including 54 female and immature elk from four different elk herds, were tested using standard *Brucella* serologic methods (card, rapid automated presumptive [RAP], and rivanol tests), as well as the western immunoblot. Samples ($n=28$) from animals known to be naturally infected with *B. abortus* biovar 1 served as positive controls. For presumed negative samples, sera ($n=26$) were collected from two elk herds in which negative serologic tests, and the absence of clinical signs of disease such as abortions, supported *Brucella*-negative classification. In addition to these study samples, serologic data from 12 tule elk (*Cervus elaphus nannodes*) were provided from the California Department of Fish and Game in order to illustrate a field application of the western blot. The western immunoblot had the highest sensitivity (1.0;% 0.899–1.0) and specificity (1.0; 0.891–1.0) among all tests used in the study. The Kappa statistic for agreement between the western blot and the card, rivanol, and RAP tests were 0.701, 0.808, and 0.921, respectively, showing good to excellent agreement with the standard diagnostic tests currently in use. Although the western immunoblot is more expensive and time intensive than other tests, in this limited study, it was shown to be reliable for establishing and confirming *B. abortus* disease status in elk. In addition to this study, subsequent applications of the western blot assay have been successful in detecting *Yersinia* sp. exposure in elk after their antibodies cross-reacted on standard *Brucella* serology.
Key words: *Brucella abortus*, *Cervus elaphus*, diagnostic test evaluation, elk, western immunoblot, *Yersinia entercolitica* O:9.

INTRODUCTION

Brucella spp. are gram-negative, facultative, intracellular bacteria which cause disease in a wide variety of domestic and wild animal species including cattle, bison (*Bison bison*), elk (*Cervus elaphus*), and moose (*Alces alces*; Creech, 1930; Thorne and Morton, 1978). In particular, *B. abortus* invades the mucous membranes of ungulates and can cause placentitis, with late-gestation abortions in females and orchitis and epididymitis in males (Bercovich, 1998). High abortion rates, decreased milk production, loss of condition, infertility, and lameness in cattle have made brucellosis extremely important to beef and milk producers around the world (Manthei and Willeberg, 1950), restricting international trade in many instances (Wilson and Beers, 2001). The eradication of the disease from the United States has been a priority of the federal government since the early 1930s, when a cooperative state-federal brucellosis program was adopted to reduce the prevalence of brucellosis in cattle; the program designated brucellosis as the most signif-

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icant livestock disease at that time. Since then, agencies have implemented a variety of livestock, wildlife, and disease risk-management strategies (Cheville and McCullough, 1998). Strict control recommendations include testing for *Brucella* prior to interstate translocation of wildlife, in addition to selection of noninfected source populations for wildlife translocations within states (Corn and Nettles, 2001). Currently, livestock in 48 states have been classified by the United States Department of Agriculture as brucellosis free. However, recent cases of cattle brucellosis in Wyoming and Montana have highlighted the challenges of managing a disease with many potential hosts and have exacerbated the tension between ranchers and wildlife managers over wildlife's potential role in the transmission of brucellosis to cattle herds (Bercovich, 1998).

Brucella diagnosis is a challenge, as no reliable ante mortem gold standard exists for determining infection status. While bacterial culture is considered 100% specific for brucellosis, it requires specific media and specialized incubation conditions, and *Brucella*'s slow growth rate often leads to overgrowth of nontarget bacteria on the culture plates. As the prevalence of brucellosis decreases in the United States, the sensitivity and specificity of diagnostic tests are becoming much more critical for appropriate agricultural and wildlife management, with the cost of a false-positive test becoming more significant. Many serologic tests have been produced to aid in diagnosing *Brucella* infections; however, all currently-used diagnostic methods were developed and validated for use in cattle. When applied to wildlife, many cattle tests have been shown to be inaccurate and unpredictable (Morton et al., 1981; Davis et al., 1990). Furthermore, antibodies developed to environmental bacteria, such as to *Yersinia enterocolitica* O:9, can cause cross-reactions in commonly used *Brucella* screening tests (Kittelberger et al., 1995). An excellent example of the challenges caused by such false-positive tests occurred in 1998, when a false-positive test result on a bull elk calf prevented the California Department of Fish and Game (CDFG) from transporting 30 Roosevelt elk captured in Oregon for translocation to California (Gonzales, pers. comm.); this animal's serum later tested positive for *Y. enterocolitica* O:9 by western blot. This example highlights the need for newer, more-specific tests in elk and bison that will perform reliably under field circumstances.

Edmonds et al. (1999) described a western immunoblot designed to differentiate antibody responses to *B. abortus*, *Brucella melitensis*, and *Brucella suis,* as well as to *Y. enterocolitica* O:9. The variation in O-antigens among the different bacterial species results in the host's development of specific antibodies to *Brucella* that can be differentiated by the western blot (Edmonds et al., 1999). To increase diagnostic capabilities for *B. abortus* infections in wildlife, the western immunoblot performance was compared to the standard *B. abortus* serologic tests in elk including the card test, the rivanol test, and the rapid automated presumptive (RAP) test. We hypothesized that the western blot would have greater specificity, while maintaining the sensitivity of standard serology, and would be a valid and useful confirmatory test for diagnosing *B. abortus* infections in elk.

MATERIALS AND METHODS

Sample selection

Sera from adult female and immature elk (*Cervus elaphus*) from four different herds were acquired from collaborators for inclusion in this study (n=54). Samples were classified as positive, or presumed negative, in order to determine the performance characteristics of the diagnostic tests in question. Two Wyoming herds that were naturally infected with *B. abortus* biovar 1 were used as positive controls (n=21 and n=7; Kreeger et al., 2002). Animals from these herds were classified as positive because they came from known *Brucella*-

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infected herds and were culture positive, or tested positive (or both), on at least three serologic tests. Elk from two California herds ($n=19$ and $n=7$) were used as presumed negative controls because all individuals came from herds with no known history of brucellosis and had never been serologically positive (data not shown). Bacterial cultures were never performed on these animals because they had never shown clinical signs, such as abortions, for brucellosis. In addition, two serologically negative samples (based on card, standard plate, rivanol, complement fixation, fluorescence polarization assay, and competitive-enzyme-linked immunosorbant assay testing; Fluegel, unpubl. data) from one of the *Brucella*-infected Wyoming herds were tested to evaluate the western blot's specificity under conditions of higher prevalence.

Sample blinding

After the samples were received from collaborators, they were randomly assigned numbers using a random number generator (http://random.org/integers). The samples and their randomly assigned numbers were recorded in a lab notebook for evaluation after the blots were read. Twelve samples from the pool of both known-positive and presumed-negative herds were evaluated blindly on each immunoblot.

Immunoblot procedure

Antigen electrophoresis and transfer to nitrocellulose was performed according to previously described methods (Schurig et al., 1991; Edmonds et al., 1999). Lysates of the individual *Yersinia* O:8, *Yersinia* O:9, *Brucella* 2308, and RB51 cultures were prepared in large lots and aliquoted for use as antigen in individual electrophoresis gels as previously described. *Yersinia* O:8 and O:9 organisms were each mixed 1:1 with sample buffer (SAB; Bio-Rad Laboratories, Hercules, California, USA) and boiled for 10 min to produce bacterial lysates. Similarly, *Brucella* 2308 and RB51 reference strains were cultured at Louisiana State University, sonicated, and then each mixed 1:1 with SAB and boiled as previously described.

The lysates were subjected to electrophoresis for approximately 1 hr at 100 V using 12% acrylamide gels (Protean II Ready Gel Precast Gel, Tris-HCl, Bio-Rad) with 1× reservoir buffer to separate the bacterial antigens. For each serum sample to be tested, five-antigen lanes were created using approximately 3 ul of blotting standard (Kaleidoscope Prestained Standards, Bio-Rad), RB51 cell lysate, 2308 cell lysate, *Yersinia* O:8, and O:9 cell lysates. Three sets of the five antigens were separated simultaneously on the 15-well gel. The separated antigens were then transferred onto nitrocellulose strips previously soaked in cold transfer buffer, using one nitrocellulose strip per 15-lane gel. The nitrocellulose strips with transferred antigens were then divided and placed into a 1:40 dilution of the serum to be tested, using Tris-buffered saline (TBS) as a diluent, covered with foil, and were incubated at room temperature on a shaker for at least 4 hr. The immunoblots were then rinsed with TBS-Tween 20 (Fisher Scientific, Pittsburg, Pennsylvania), followed by a final rinse with 1× TBS, and then incubated for 45 min with a 1:800 dilution of rabbit anti-bovine IgG–horseradish peroxidase (HRP) conjugate (Sigma Chemical Company, St. Louis, Missouri, USA) in 1× TBS (10 mls per membrane). Finally, the immunoblots were developed using a methanol-chloronapthol with TBS-hydrogen peroxide mixture (Edmonds et al., 1999).

As described previously, the separation of *B. abortus* and *Y. enterocolitica* O:9 antigens produced a nondiscrete laddering pattern of protein segments between 29 and 68 kDa molecular weight (MW) (Schurig et al., 1991; Edmonds et al., 1999). *Brucella* seropositive serum samples were characterized by four major bands between 29 to 68 kDa MW, with additional indiscrete bands and staining from the high through the low MW regions (based on protein standards run concurrently on the same gel) in the 2308 lane, caused by binding of anti-O-polysaccharide antibodies to separated antigen protein segments (Edmonds et al., 1999). The four distinct protein bands and characteristic staining pattern were necessary for a sample to be classified as positive. A small percentage (<5%) of negative serum samples showed some background staining between 29 and 68 kDa MW in the 2308 lane, but only positive samples showed the four distinct bands and complete protein ladder. *Yersinia enterocolitica* O:9 seropositive samples showed a homologous banding and color pattern between 29 and 68 kDa MW in the O:9 lane due to the binding of the O-polysaccharide homopolymer, but without the distinct protein banding pattern in the 2308 lane. In situations where background color development of the immunoblot obscured clear visualization of the antigen pattern, the test results were considered indeterminate. Each immunoblot was performed in duplicate. If the duplicates were not in agreement, a third test was performed to confirm the result. In the case of an indeterminate test, the sample was rerun until

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TABLE 1. Sensitivity and specificity estimates (with 95% exact binomial confidence intervals, or 95% CIs) and percent indeterminate test results for card, rivanol, rapid automated presumptive (RAP), and western immunoblot tests used to detect *Brucella abortus* infection in serum samples from 28 confirmed positive and 26 presumed negative elk (*Cervus elaphus*).

Test	Sensitivity % (95% CI)	Specificity % (95% CI)	% Indeterminant
Card	96.4 (81.7–99.9)	76.9 (56.4–91.0)[a]	0
Rivanol	80.8 (60.6–93.4)[b]	100.0 (89.1–100.0)	0
RAP	96.4 (81.7–99.9)	95.7 (78.1–99.9)	5.4
Western blot	100.0 (89.9–100.0)	100.0 (89.1–100.0)	0[c]

[a] Differs from specificity of rivanol and western blot (McNemar's χ^2, $0.025<P<0.050$).

[b] Differs from sensitivity of western blot (McNemar's χ^2, $0.025<P<0.050$).

[c] Indeterminant blots rerun until an interpretable result was obtained.

a definitive test result was determined. For *Brucella*-seronegative samples, *Yersinia* sp. exposure was also evaluated by looking for immunoreactivity in the *Y. enterocolitica* O:9 and O:8 lanes.

Standard *Brucella* agglutination testing

All serum samples were evaluated using standard *B. abortus* serologic tests according to standard laboratory protocols (Mikolon et al., 1998). Card, rivanol, and RAP tests were selected because they are often used to screen wild elk for brucellosis exposure and require serum volumes of less than 0.5 ml (Al Dahouk et al., 2003; United States Department of Agriculture [USDA] Animal and Plant Health Inspection Service, 2003). The testing was performed per the USDA Uniform Methods and Rules (Document 91-45-013, USDA Animal and Plant Health Inspection Service, 2003) at the California Animal Health and Food Safety (CAHFS) Laboratory by USDA-approved technicians.

Statistical analysis

The immunoblot results were compared with standard *B. abortus* serologic assays. A Kappa statistic was calculated for each comparison as a measure of agreement between duplicate western blot runs of a single sample, as well as between immunoblot and other test methodologies (Cohen, 1960). Kappa statistics were also calculated for *Y. enterocolitica* O:9 results for the presumed *Brucella*-negative samples between duplicate western blot runs of each sample. Sensitivity (Se) and specificity (Sp), with 95% confidence intervals, were calculated using exact binomial methods (Greiner and Gardner, 2000) with MINITAB software (State College, Pennsylvania, USA). McNemar's χ^2 test was used to compare the Se and Sp among the scientific tests (McNemar, 1947). Because false positive and negative tests are of major concern with *Brucella* serology, positive predictive value (PPV) and negative predictive value (NPV) were also calculated using the Se and Sp for the specific tests, assuming either 50% or 1% prevalence (Gordis, 2004), in order to simulate *Brucella*-endemic populations and populations where the disease is close to eradication, respectively.

Field application

In addition to the study samples described previously, samples ($n=12$) from a 2005 CDFG elk capture were also evaluated at Louisiana State University (LSU; Dr. P. Elzer) to assess the performance of the immunoblot in a diagnostic laboratory situation. After these elk tested positive for *Brucella* with standard serologic screening tests, the samples underwent further diagnostic testing, including submission to CAHFS for complement fixation, standard plate, and buffered acidified plate antigen testing as well as for immunoblot evaluation at LSU.

RESULTS

The western immunoblot proved to have 100% Se and Sp for the known-status elk tested (Table 1). The western blot also identified as negative two serum samples from an infected Wyoming herd that were serologically negative on five other established serologic tests. However, five out of the 133 total blots analyzed (3.8%) could not be discriminated by visual observation and were therefore classified as indeterminate.

The Kappa statistic for agreement between the western blot and the card,

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TABLE 2. Positive predictive values (PPV) and negative predictive values (NPV) for *Brucella abortus* testing using card, rivanol, rapid automated presumptive (RAP), and western immunoblot tests based on experimentally-determined sensitivities and specificities from 54 serum samples from elk (*Cervus elaphus*) in this study for two different prevalences in hypothetical elk herds.

Test	50% Prevalence		1% Prevalence	
	PPV	NPV	PPV	NPV
Card	80.7%	96.5%	4.0%	100.0%
Rivanol	100.0%	83.9%	100.0%	99.8%
RAP	95.7%	96.5%	18.5%	100.0%
Western blot	100.0%	100.0%	100.0%	100.0%

rivanol, and RAP tests were 0.701, 0.808, and 0.921, respectively. The duplicate western blot analyses also had excellent agreement, with a Kappa statistic of 0.822. In comparing Se and Sp between tests, the only parameters that showed a significant difference were the Sp between the card test and the rivanol test-western blot (McNemar's $\chi^2 = 6$, $0.025 < P < 0.050$) and the Se between the rivanol test and the western blot (McNemar's $\chi^2 = 5$, $0.025 < P < 0.050$).

In order to establish confidence in test results for *Brucella* diagnosis under different circumstances, PPV and NPV were calculated for two different hypothetic prevalences of *B. abortus*—50% and 1% (Table 2). The western blot had the highest NPV overall (100%) and maintained 100% PPVs, equal to those of the rivanol test.

Brucella antibody-positive samples showed homologous banding and color staining in both the *B. abortus* and *Y. enterocolitica* O:9 lanes, obscuring the ability of the western blot to differentiate the presence or absence of *Y. enterocolitica* O:9 antibodies. Of the 24 presumed negative samples, 16 showed variable immunoreactivity against *Y. enterocolitica* O:9 for at least one immunoblot. The Kappa statistic for *Y. enterocolitica* O:9 results was 0.253, showing poor agreement between duplicate immunoblots on the same sample.

Laboratory testing of the 2005 CDFG elk capture samples showed variable results on brucellosis serology (Table 3). Four animals tested positive on all standard serologic tests run. However, all animals were positive for *Yersinia* antibodies and negative for *B. abortus* antibodies on the immunoblot.

DISCUSSION

Translocation has become increasingly important to the maintenance of wild herds of ungulates due to habitat fragmentation and concerns over genetic diversity and adequate hunting capacity. With lower herd densities and increasing budget pressures, efficiency of captures and disease surveillance has become paramount to wildlife management agencies. Therefore, accurate diagnostics are critical to ensure appropriate focus on finding the best ungulate source populations, based on genetics and herd dynamics rather than on finances. Unfortunately, inappropriate or unvalidated diagnostic tests have hampered the management effectiveness of wild ungulate herds, especially in the absence of clinical signs of infectious diseases.

In general, serologic screening for infectious diseases can be extremely time and cost efficient and may allow for retrospective evaluation of pathogen prevalence. However, the efficiency of these methods is negated by incorrect results (due to suboptimal sensitivity and specificity values), potentially leading to misinformed management decisions. Many serologic tests provide spurious results because of cross-reactivity between structurally or biochemically similar organisms,

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TABLE 3. Serology and blot results from a 2005 tule elk (*Cervus elaphus nannodes*) capture in California. Diagnostics include the complement fixation test (CF), the standard plate agglutination (SPA) test, the buffered antigen plate agglutination (BAPA) test, the rivanol test, and the western immunoblot.

					Western blot	
Animal ID	CF	SPA	BAPA	Rivanol	*Yersinia*	*Brucella*
1	+++[a]	(−)	(−)	IU[b]	weak +	(−)
2	+	+	+	+	+	(−)
3	+++	+	+	+	+	(−)
4	+++	+	+	+	++	(−)
5	IU	(−)	(−)	IU	+	(−)
6	(−)	+	+	+	+	(−)
7	(−)	+	+	+	+	(−)
8	(−)	+	+	+	++	(−)
9	IU	(−)	(−)	IU	++	(−)
10	++++	+	+	+	++	(−)
11	IU	+	+	+	++	(−)
12	IU	(−)	(−)	IU	+	(−)

[a] (−) = negative; + = mildly positive; ++ = moderately positive; +++ = strongly positive; ++++ = extremely positive.
[b] IU = information unavailable.

such as *B. abortus* and *Y. enterocolitica*, and nonexistent or incomplete validation of the tests in the species of interest. Through species-specific validation of diagnostic tests, and by selectively using highly sensitive screening tests followed by more specific confirmatory tests, the diagnostician and wildlife manager can be reasonably comfortable with the information on which management decisions will be based. In the specific case of *Brucella* testing, the difficulties with culture have resulted in the lack of a true "gold standard" to detect infection. Serology provides the best opportunity for successful and accurate diagnosis of *B. abortus* infections.

In this study, the immunoblot procedure performed competitively with, and exceeded the diagnostic capability of, standard *Brucella* serology in elk. It has a sensitivity comparable to standard agglutination tests with higher specificity. As disease prevalence decreases, the immunoblot maintains a very high positive and negative predictive value, which is extremely valuable for testing elk in areas where eradication of brucellosis is being attempted, or where *Brucella* infections are already rare. In a hypothetical area with 50% disease prevalence, based on the results of this study, testing 2,000 animals with the card, rivanol, and RAP tests will yield 36, 192, and 36 false-negative tests and 231, 0, and 43 false-positive tests, respectively. The immunoblot, when run in duplicate, produced no false-positive or -negative tests in this study. Having a test that maintains the sensitivity of standard serology, yet is more specific, will help prevent crucial management mistakes and avoid problems caused by false-positive tests.

The immunoblot does have technical drawbacks, however. It requires a highly trained technical staff and, at least in our hands, returned a number of indeterminate tests when first being established in the laboratory. Average time for completion of a test run was approximately 12 hr for a set of 12 immunoblots and 30 min or less for the standard serology. The immunoblot is also more expensive than standard serology, with card, rivanol, and RAP tests costing under US $5.00 per sample at diagnostic laboratories (inclusive of personnel time) and the immunoblot costing approximately US $10.00 per sample in consumables alone. Further assay development to allow for automation and cost

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reduction seems warranted, based on initial findings. Immunoblot results for the presumed *Brucella*-negative animals, however, shows that *Yersinia* results may be variable between duplicate immunoblots on the same serum sample, and further work is needed to assess the ability of the immunoblot to provide differential diagnosis for *Yersinia* infection.

Based on these factors, the immunoblot may be most useful as a confirmatory test for brucellosis—after elk test positive on faster, less expensive screening tests. Results from the 2005 CDFG elk capture provided excellent evidence for the usefulness of the immunoblot procedure in a real-world setting. In fact, given the prior *Brucella*-free disease status from California, coupled with the immunoblot results, the USDA was satisfied in diagnosing the elk as *Brucella*-free and *Yersinia*-positive. This example illustrates the usefulness of the immunoblot procedure as a confirmatory test in areas of low prevalence. While results varied widely among the different serologic tests available for predicting *Brucella*-*Yersinia* status, the immunoblot was the only test that reliably differentiated between these exposures in elk not infected with *Brucella*. Additional work should be undertaken, both on free-ranging elk as well as on other free-ranging ungulate species, to more fully explore the utility of this method to confirm or refute *Brucella* status when the incorrect results of other tests can lead to catastrophic wildlife management results.

ACKNOWLEDGMENTS

This research was made possible, in part, by a competitive grant from the University of California, Davis Wildlife Health Center. The authors would also like to thank the California Department of Fish and Game, the Wyoming Department of Game and Fish, the United States Fish and Wildlife Service, and the San Luis National Wildlife Refuge for sample acquisition. We would also like to acknowledge the technical staff at the California Animal Health and Food Safety Laboratory for sample processing and H. Edwards and staff at the Wyoming State Veterinary Laboratory for further assistance. Finally, we would like to thank S. Hagius and the staff at the Elzer laboratory at Louisiana State University for being a tremendous resource at all stages of this study.

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Received for publication 12 September 2008.

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