**Evaluation of Enzyme-Linked Immunosorbent Assays for Detection of Mycoplasma bovis-Specific Antibody in Bison Sera**

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Mycoplasma bovis has recently emerged as a significant and costly infectious disease problem in bison. A method for the detection of *M. bovis*-specific serum antibodies is needed in order to establish prevalence and transmission patterns. Enzyme-linked immunosorbent assays (ELISAs) validated for the detection of *M. bovis*-specific serum IgG in cattle are commercially available, but their suitability for bison sera has not been determined. A collection of bison sera, most from animals with a known history of infection or vaccination with *M. bovis*, was tested for *M. bovis*-specific IgG using commercially available kits as well as an in-house ELISA in which either cattle or bison *M. bovis* isolates were used as a source of antigen. Comparison of the results demonstrates that ELISAs optimized for cattle sera may not be optimal for the identification of bison seropositive for *M. bovis*, particularly those with low to moderate antibody levels. The reagent used for the detection of bison IgG and the source of the antigen affect the sensitivity of the assay. Optimal performance was obtained when the capture antigen was derived from bison isolates rather than cattle isolates and when a protein G conjugate rather than an anti-bovine IgG conjugate was used for the detection of bison IgG.

**MATERIALS AND METHODS**

**Bison sera.** Fifty-five serum samples from bison were available for testing, characterized as falling into one of four groups, as follows: group 1, sera collected from healthy free-range bison 3 to 6 weeks after immunization with an experimental *M. bovis* bacterin; group 2, sera collected 2 to 4 weeks after experimental infection of healthy captive bison with *M. bovis*; group 3, sera collected from free-range or outdoor captive bison euthanized due to *M. bovis*-related respiratory disease; and group 4, sera collected from healthy free-range or captive bison with an uncertain history of exposure to *M. bovis* (Table 1). The sera represent samples from a total of 46 bison; 9 bison in group 4 were also the sources of subsequently obtained samples assigned to groups 1 or 2.

**Commercial ELISAs.** Commercially available ELISA kits for detection of *M. bovis*-specific antibodies in cattle were obtained from Bio-X Diagnostics (Jemelle, Belgium) and Biovet (Saint-Hyacinthe, Quebec, Canada). Each assay was carried out and results were interpreted according to the respective manufacturer’s recommendations. Where indicated, protein G-peroxidase (Pierce; diluted 1:1,000) was substituted for the anti-bovine IgG-peroxidase conjugate supplied with the Biovet kit.

**M. bovis isolates.** M23 was the *M. bovis* cattle isolate initially selected for antigen production, based on its demonstrated performance as a source of broadly cross-reactive ELISA antigen that provides sensitive and reproducible detection of seropositive cattle (61; R. Rosenbusch, personal communication). Two additional cattle isolates, F148 and 94605 (7), were used to prepare antigen for testing of selected sera, as detailed below. Three bison isolates of *M. bovis* that were acquired between 2007 and 2011, two from the United States and one from Canada, from animals with respiratory disease attributable to no other etiology served as the source of a bison isolate ELISA antigen cocktail. The isolates represent all genotypes known to infect bison, as defined by MLST (L. Thole and K. B. Register, presented at the Merial-NIH National Veterinary Scholars Symposium, Fort Collins, CO, 2 to 5 August 2012). A method for the detection of *M. bovis*-specific antibodies in cattle was obtained from Bio-X Diagnostics (Jemelle, Belgium) and Biovet (Saint-Hyacinthe, Quebec, Canada). Each assay was carried out and results were interpreted according to the respective manufacturer’s recommendations. Where indicated, protein G-peroxidase (Pierce; diluted 1:1,000) was substituted for the anti-bovine IgG-peroxidase conjugate supplied with the Biovet kit.

**In-house ELISA.** Isolates of *M. bovis* used for in-house ELISA antigen production were grown for 18 to 24 h at 37°C in PPLO broth supplemented with 10 g/liter yeast extract and 20% horse serum, in an atmosphere of 5% CO₂. Bacteria were pelleted and washed three times by centrifugation at 12,000 × g for 20 min, in a 10× volume of phosphate-buffered saline, pH 7.2, containing 0.02% NaN₃.

**Commercial ELISAs.** Commercially available ELISA kits for detection of *M. bovis*-specific antibodies in cattle were obtained from Bio-X Diagnostics (Jemelle, Belgium) and Biovet (Saint-Hyacinthe, Quebec, Canada). Each assay was carried out and results were interpreted according to the respective manufacturer’s recommendations. Where indicated, protein G-peroxidase (Pierce; diluted 1:1,000) was substituted for the anti-bovine IgG-peroxidase conjugate supplied with the Biovet kit.
TABLE 1 Summary of ELISA results with commercial and in-house assays

| Serum source (n)                                      | No. of sera | In-house assaysa | Commercial assays | Biovet with protein G conjugateb |
|------------------------------------------------------|-------------|------------------|-------------------|----------------------------------|
| Group 1, immunized (12)                              | 2           | Positive Positive | 4+ 1+ NT          |                                  |
|                                                       | 1           | Positive Positive | 4+ Negative 4+    |                                  |
|                                                       | 1           | Positive Positive | 3+ 2+ NT          |                                  |
|                                                       | 1           | Positive Positive | 3+ 1+ 3+         |                                  |
|                                                       | 2           | Positive Positive | 3+ Negative 3+    |                                  |
|                                                       | 1           | Positive Positive | 2+ 1+ NT          |                                  |
|                                                       | 2           | Positive Positive | 2+ Negative 1+    |                                  |
|                                                       | 1           | Positive Positive | 2+ Negative 2+    |                                  |
| Group 2, experimentally infected (14)                 | 1           | Positive Positive | 5+ 3+ NT          |                                  |
|                                                       | 1           | Positive Positive | 5+ 2+ NT          |                                  |
|                                                       | 3           | Positive Positive | 5+ 1+ NT          |                                  |
|                                                       | 2           | Positive Positive | 4+ 1+ NT          |                                  |
|                                                       | 2           | Positive Positive | 4+ Negative 4+    |                                  |
|                                                       | 1           | Positive Positive | 3+ 1+ NT          |                                  |
|                                                       | 1           | Positive Positive | 1+ Negative 3+    |                                  |
|                                                       | 1           | Positive Positive | 2+ 1+ NT          |                                  |
|                                                       | 1           | Positive Positive | 2+ Negative 3+    |                                  |
| Group 3, with respiratory disease (15)                | 1           | Positive Positive | 5+ 4+ NT          |                                  |
|                                                       | 1           | Positive Positive | 5+ 3+ NT          |                                  |
|                                                       | 2           | Positive Positive | 5+ Negative 4+    |                                  |
|                                                       | 1           | Positive Positive | 4+ 3+ 4+         |                                  |
|                                                       | 2           | Positive Positive | 3+ 1+ NT          |                                  |
|                                                       | 2           | Positive Positive | 3+ Negative 4+    |                                  |
|                                                       | 1           | Positive Positive | 2+ 1+ 4+         |                                  |
|                                                       | 1           | Positive Positive | 2+ Negative 4+    |                                  |
|                                                       | 1           | Positive Positive | 1+ Negative 4+    |                                  |
|                                                       | 1           | Positive Positive | 1+ Negative 1+    |                                  |
|                                                       | 1           | Positive Positive | 1+ Negative 4+    |                                  |
| Group 4, healthy, history unknown (14)                | 1           | Positive Positive | 3+ 2+ NT          |                                  |
|                                                       | 2           | Positive Positive | 2+ Negative Negative |                              |
|                                                       | 6           | Positive Positive | 1+ Negative Negative |                              |
|                                                       | 1           | Positive Positive | 1+ Negative 3+    |                                  |
|                                                       | 2           | Positive Positive | 1+ Negative 1+    |                                  |

a Source of isolates used for antigen production; the bison in group 2 were infected with the same 3 bison isolates as used for antigen production.

b Positive results were classified as 1+ to 5+ according to the kit protocol.

c Positive results were classified as 1+ to 4+ according to the kit protocol.

d Protein G-peroxidase conjugate was substituted for the anti-bovine IgG-peroxidase conjugate provided with the kit.

e NT, not tested.

buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.4). Tween 20-soluble proteins were extracted using a previously reported method (8), and total protein was quantitated using a detergent-compatible, commercially available kit (Bio-Rad). The 3 M. bovis bison isolates serving as the source of the ELISA antigen cocktail were grown separately and used to prepare individual Tween 20 extracts, which were then combined in equivalent amounts (in μg/ml) for use as bison isolate antigen. Tween 20 extracts were diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6 (Sigma), such that 0.5, 1, 2, or 4 μg per well, in 100 μl of solution, was delivered to each of three different 96-well plates evaluated (Immulon 1B, Immulon HB, and Nunc MaxiSorp). Plates were sealed and incubated at 37°C for 3 h, followed by 3 washes with Tris-buffered saline-Tween (TBST) (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) containing 0.1% bovine serum albumin (BSA). One hundred microliters of blocking solution (TBST with 1% BSA) was added to each well, and plates were incubated for 2 h at room temperature and then washed 3 times as described above. Each plate was tested with 1:50, 1:100, and 1:200 dilutions (prepared in wash buffer) of control sera. Serum from a healthy bison calf born in captivity to a healthy cow from a herd with no history of infection with M. bovis (both housed at the National Animal Disease Center) was used as a negative control. The source of bison serum used as a positive control was an animal that had been experimentally infected intranasally with M. bovis; lung lesions typical of mycoplasmosis in bison (2, 3) were apparent at necropsy, 4 weeks later (K. B. Register, S. C. Olsen, R. E. Sacco, J. F. Ridpath, S. M. Falkenberg, and R. J. Madison, unpublished data). Following 3 washes, 100 μl of protein G-peroxidase conjugate was substituted for the anti-bovine IgG-peroxidase conjugate provided with the kit.
plates were incubated for 30 min at 37°C. After 3 additional washes, 100 µl of the Pierce anti-bovine IgG-peroxidase conjugate (Pierce; tested at both 1:1,000 and 1:10,000) was added to each well and substrate reaction time of 15 min. These conditions were used for all subsequent in-house ELISAs. Color development was halted after 15 min with the addition of ABTS peroxidase stop solution (KPL, Inc.). Results from this optimized assay were defined as an average absorbance greater than the average plus 3 standard deviations for the negative control, calculated independently for each plate analyzed (values ranged from 0.259 to 0.374). The basis for comparisons between sera testing positive was the average sample absorbance minus the positive cutoff value (ΔA). Student’s two-tailed t test was used to evaluate the statistical significance of differences in ΔA.

Immunoblots. The M23 and bison isolate antigen preparations used for the in-house ELISA were also used for Western blotting. Proteins (15 µg per lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% dried milk in TBST and were incubated for 1 h at room temperature with the specified sera, diluted as indicated in blocking solution. After three washes with TBST, membranes were incubated for 1 h at room temperature with protein G-peroxidase (Pierce; diluted 1:2,000 in blocking solution) and then washed three times in TBST. The Pierce ECL Plus Western blotting substrate (Thermo Scientific) was used for detection.

RESULTS AND DISCUSSION

At present, two commercially available ELISA kits have been validated for detection of M. bovis-specific antibodies in cattle, both of which incorporate one or more recombinant M. bovis proteins as the capture antigens. Both assays also define intermediate levels of positivity, i.e., 1 to 5+ for the Bio-X ELISA and 1+ to 4+ for the Biovet assay. The Bio-X ELISA includes proteins M. bovis G-peroxidase for detection of bound antibody, while the Biovet assay utilizes an anti-bovine IgG-peroxidase conjugate. The affinity of protein G for bison IgG has been reported as equivalent to that for bovine IgG (9) but, at the time this work was undertaken, no information was available regarding the affinity of anti-bovine IgG for bison IgG.

The recombinant M. bovis proteins used as capture antigens in commercially available ELISAs are encoded by genes from cattle isolates. The observation that bison isolates are genetically distinct from all cattle isolates so far evaluated (L. Thole and K. B. Register, presented at the Merial-NIH National Veterinary Scholars Symposium, Fort Collins, CO, 2 to 5 August 2012) raises concerns regarding whether ELISA capture antigens derived from cattle isolates provide optimal sensitivity for detection of seropositive bison. To address this point, an in-house ELISA was developed, and its basic parameters were optimized (as described in Materials and Methods), so that direct comparisons of assays with capture antigen originating from cattle versus bison isolates could be made under otherwise identical conditions.

All sera were tested in both commercial ELISA formats and with the in-house ELISA using cattle isolate antigen and bison isolate antigen. Results are summarized in Table 1. All sera were positive with the in-house ELISA, regardless of the source of antigen used. The average ΔA values for the positive-control serum were 0.851 when tested with antigen from isolate M23 and 1.836 when tested with bison isolate antigen. Results from the Bio-X assay were largely congruent with those of the in-house assay, with only 2 samples being identified as negative, both obtained from bison with an uncertain history of exposure to M. bovis. Among the Bio-X assay-positive samples, 17 were classified as either 4+ or 5+, the two highest levels of positivity defined by the assay. In contrast, 22 sera were positive with the Biovet assay, including only 21/41 bison in groups 1 to 3, with a history of infection or vaccination with M. bovis. Only 4 Biovet assay-positive samples reached one of the two highest levels of positivity. An obvious distinguishing feature of the Biovet assay, compared to the other assays, is an inclusion of an anti-bovine IgG conjugate rather than a protein G conjugate for detection of IgG. When protein G-peroxidase was substituted for anti-bovine IgG-peroxidase from the Biovet kit, all except one of the sera from bison in groups 1 to 3 that previously had tested negative instead tested positive, most at the level of 3+ or 4+ (Table 1). Additionally testing positive were 4/11 sera from group 4 that were negative with the Biovet anti-bovine IgG conjugate but positive in all other ELISA formats. These data suggest that the Biovet ELISA designed for use in cattle may be unsuitable for bison sera due, at least in part, to limited binding of the anti-bovine IgG conjugate in the kit to bison IgG.

Anti-bovine IgG-peroxidase from a different source (KPL) also appeared to bind poorly to bison IgG, compared to protein G-peroxidase, based on results with the bison positive-control serum using the in-house ELISA. In agreement with these observations, Pruvo et al. (10) recently reported that purified bison IgG binds poorly or not at all to anti-bovine IgG conjugates from two different sources.

Because of the different scales of positivity applied to data from the Bio-X and Biovet/protein G assays, a straightforward intersay comparison of the results from sera testing positive in both assays is problematic. Additionally, there was no attempt to validate the Biovet assay with the protein G conjugate or to optimize the conjugate dilution used. However, under the conditions employed, it was apparent that a number of sera could not be consistently defined as either strongly or weakly positive. Several samples classified as 3+ or 4+ positive with the Biovet/protein G assay were only weakly positive (1+ or 2+) with the Bio-X ELISA. Although sera that were strongly positive with the Bio-X assay and were also evaluated using the Biovet/protein G format were strongly positive in both instances, only 6 of 17 such samples were dually tested. Considering sera from animals in group 4, with an uncertain history of exposure to M. bovis, those that were weakly positive with the Bio-X assay ranged from negative to 3+ positive when evaluated with the Biovet/protein G method. Differences in assay parameters and reagents, such as the number, identity, and concentration of the recombinant M. bovis proteins employed as antigen and the substrate used, likely account for at least some of these discrepancies.

While the source of antigen included in the in-house ELISA did not affect the overall result, the average ΔA values were higher for 52/55 sera when bison isolate antigen was used; for 35 sera, the difference was statistically significant (P ≤ 0.05), as was the difference in a group-wise comparison of the average ΔA values for all sera (0.990 with bison isolate antigen versus 0.668 with M23 antigen; P = 1.86 × 10−9). Different reactivity levels depending on the antigen source also were apparent when sera were evaluated by immunoblotting. A serum specimen in group 2 (Table 2, serum B) for which the ΔA value was significantly higher with bison isolate antigen bound to multiple proteins in that preparation but had...
little detectable reactivity with M23 antigen at the dilution used
(Fig. 1A, lanes 3 and 4). A serum specimen in group 3 whose
ΔA value was not significantly affected by the antigen source reacted
with multiple proteins in both antigen preparations (Fig. 1A, lanes
5 and 6). Although a larger number of proteins were reactive in the
bison isolate antigen preparation, the collective intensity of bands
appeared only slightly greater than that for antigen prepared from
isolate M23, consistent with the corresponding ΔA values (1.87
versus 1.59). Immunoblots further revealed that patterns of reac-
tivity with the bison isolate antigen were not consistent among
sera (Fig. 1A, lane 4 versus lane 6), which is indicative of antigenic
heterogeneity among bison isolates.

Although M23 was isolated ~20 years ago, it remains a reliable
source of ELISA antigen for detection of seropositive cattle (R.
Rosenbusch, personal communication), suggesting that its anti-
genic profile is not greatly dissimilar from that of M. bovis isolates
currently circulating in livestock. Nonetheless, antigenic drift over
time, leading to an antigenically divergent population, might ex-
plain the different levels of reactivity of bison sera with M23 versus
the bison isolate antigen cocktail. To address this possibility, the
reactivity of a subset of 6 bison sera with Tween 20 extracts from
two recent cattle isolates, acquired in 2010 from different coun-
tries, was evaluated using the in-house ELISA, and findings were
compared to results obtained with bison isolate antigen. The sera
selected included 2 each that tested 1+, 2+, and 3+ positive in the
Bio-X assay. With one exception (serum E tested with antigen
from cattle isolate 94605; P = 0.099), all sera exhibited signifi-
cantly higher average ΔA values with bison isolate antigen than
with any cattle isolate antigen tested (Table 2). In group-wise
comparisons, average ΔA values for results with bison isolate anti-
gen were significantly higher than those for all other groups.
Among cattle isolates, antigen derived from strain 94605 provided
a significant increase in the average ΔA value, compared to the
other isolates tested. These data suggest that antigen derived from
bison isolates will likely afford a higher level of sensitivity for de-
tection of M. bovis-specific antibodies in bison sera and there may
be considerable variability in the results obtained with antigen
produced from different cattle isolates. Information in Table 2
additionally suggests that the bison isolate antigen cocktail uti-
lized here for the in-house ELISA is suitable for widespread use,

### TABLE 2 Effect of ELISA antigen source on ΔA

| Serum sample | ΔA with antigen from: | Cattle isolate | Bovine isolate 
|--------------|----------------------|---------------|----------------------
|              | Bovine isolate cocktail | M23 | F148 | 94605 |
| A            | 1.744b               | 0.614 | 0.629 | 0.884 |
| B            | 1.951b               | 1.000 | 0.836 | 1.154 |
| C            | 2.022b               | 1.159 | 1.480 | 1.692 |
| D            | 1.476b               | 0.759 | 0.777 | 1.198 |
| E            | 1.717               | 1.194b | 1.333b | 1.606 |
| F            | 1.271b               | 0.282 | 0.193 | 0.623 |
| Group average | 1.697c              | 0.835 | 0.875 | 1.193d |

- a P ≤ 0.016 versus average ΔA for the same serum using antigen from all other sources indicated.
- b P ≤ 0.007 versus average ΔA for the same serum using bison isolate antigen cocktail.
- c P ≤ 0.0006 versus group average ΔA using antigen from all other sources indicated.
- d P ≤ 0.001 versus M23 and F148 antigen.

antigen cocktail was observed in both ELISA and Western blots
not only with sera from bison in group 2 (Table 2, sera A, B, and C,
and Fig. 1A, lane 4), which were infected with the same M. bovis
isolates as those used as the source for antigen preparation (Table
1), but also with sera from bison in group 3 (Table 2, sera D and E,
and Fig. 1A, lane 6) and group 4 (Table 2, serum F), which were
naturally infected with M. bovis isolates of unknown origin. Fur-
ther, the difference in the average ΔA for all sera from group 2,
compared to that of group 1 (1.154 and 0.959, respectively), was
not statistically significant (P = 0.28); the average ΔA for sera
from group 3 (1.396) exceeded that for group 2.

As discussed above, when results were considered only as posi-
tive or negative, there was a strong correlation between the
in-house ELISA and Bio-X assay results, but two sera from bison
with an unknown history of exposure to M. bovis were positive only
with the in-house ELISA (ΔA values of 0.110 to 0.182, depending
on the serum and the antigen source). Comparison of the
in-house ELISA absorbance values for those sera with values for
the negative control indicated a high probability that the sera were,
in fact, positive for M. bovis-specific antibody (P ≤ 0.0016 for both
sera, regardless of the antigen used). Results of immunoblotting
also were consistent with weak positivity (Fig. 1B). Proteins reac-
tive with those sera but not with the negative-control serum were
evident with both M23 antigen (Fig. 1B, lane 1 versus lanes 3
and 5) and the bison isolate antigen (Fig. 1B, lane 2 versus lanes
4 and 6). It also should be noted that the Bio-X ELISA antigen
consists of a single recombinant M. bovis protein, selected on the
basis of broad reactivity with sera from infected cattle. Whether the
specificity of the serum antibody response to M. bovis in bison mimics
that of cattle is unknown, but the host species-specific differential
reactivity of the bison sera examined here suggests that significant
differences may exist. Moreover, of the 20 sera most strongly re-
active with the in-house ELISA and bison isolate antigen (average
ΔA = 1.641), 12 were only weakly or moderately positive (1+, 2+,
or 3+) with the Bio-X assay.

In summary, the data presented suggest that ELISAs optimized
for detection of M. bovis-specific serum IgG in cattle may not be
optimal for identification of seropositive bison, particularly those
with low to moderate antibody levels. Both the reagent used for
detection of bison IgG and the source of the antigen affect the
sensitivity of the assay. The in-house ELISA developed in the
course of this study performed with 100% sensitivity with respect to bison known to have been infected or vaccinated with *M. bovis* and provides a basis for development of a standardized and highly sensitive clinical and epidemiological tool. Further refinement of the method requires the identification of additional seronegative bison to serve as sources for a representative negative-control pool. Serum from only one bison available for this study was reproducibly negative in all of the ELISA formats tested. Use of cattle sera cannot be recommended, since the possible effects of host species-related differences in nonspecific binding of serum components are unknown.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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