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Antibody Responses of Cervids (*Cervus elaphus*) following Experimental *Mycobacterium bovis* Infection and the Implications for Immunodiagnosis[▽]

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Captive and free-ranging wildlife animals are implicated in the maintenance and transmission of bovine tuberculosis and therefore pose a significant obstacle to eradication of the disease from domestic livestock. The current antemortem diagnostic method, the intradermal tuberculin skin test, is impractical for routine use with many wild animals. Antibody-based assays are particularly attractive because the animals are handled only once and immediate processing of the sample is not required. This report characterizes the antibody responses of red deer-elk hybrids (*Cervus elaphus*) against *Mycobacterium bovis* and subsequently evaluates the diagnostic performance of select antigens in a rapid-test format. Sequential serum samples were collected from 10 animals experimentally infected with *M. bovis* and 5 noninfected animals over a 7-month period postinfection (p.i.). Samples were evaluated by enzyme-linked immunosorbent assays, immunoblot analyses, and multiantigen print immunoassays for seroreactivity to mycobacterial antigens. Although all infected animals produced antibodies to *M. bovis* protein antigens, there was significant animal-to-animal variation in the kinetics and magnitudes of responses and the antigens recognized. The most frequently recognized antigens included MPB83, ESAT-6, CFP10, and MPB70. Responses to some antigens, such as MPB83, were consistently detected as early as 4 weeks after inoculation, whereas other antigens were detected only much later (>140 days p.i.). Antibody responses were boosted by injection of tuberculin for intradermal tuberculin skin testing. Comparison of single-antigen (fluorescence polarization assay) with multiantigen (CervidTB STAT-PAK) rapid tests demonstrated that a highly sensitive and specific serodiagnostic test for tuberculosis in cervids will require multiple and carefully selected seroreactive antigens covering a broad spectrum of antibody specificities.

Mycobacterium bovis, the etiological agent of bovine tuberculosis (TB), has an extraordinarily broad mammalian host range that includes humans, domestic livestock, and wildlife. Globally, several wildlife species have been implicated in the maintenance and transmission of *M. bovis*. In North America, these include white-tailed deer (*Odocoileus virginianus*) in northeastern Michigan (37), elk (*Cervus elaphus*) in and around Riding Mountain National Park, Manitoba, Canada (31), and wood bison (*Bison bison athabascae*) in and around Wood Buffalo National Park, Alberta, Canada, and the Northwest Territories (31). The continued transmission of *M. bovis* from free-ranging wildlife reservoirs to domestic livestock results in significant economic losses and is a significant barrier to the success of national eradication and control programs (5). Similarly, TB agent-infected captive wildlife maintained in farms or animal parks (e.g., zoos or game farms) may also serve as foci of infection for domestic animals, free-ranging wildlife, and humans (7, 42). The existing methods for diagnosing bovine TB that rely on cell-mediated immune reactivity are either inadequate, not fully developed, or not validated for use in wildlife. The most frequently employed antemortem test, the tuberculin skin test, involves the intradermal administration of a tuberculin which is composed of poorly defined mycobacterial antigens, some of which are highly cross-reactive with other environmental nontuberculous *Mycobacterium* spp. (29). Other limitations of this test include anergic responses in animals with high bacterial loads (20) and a requirement for handling animals twice over a 72-hour period. The additional handling increases the risk of capture-associated injuries to which wildlife are particularly prone.

For these reasons, alternative diagnostic approaches that are both accurate and efficient are required. A stand-alone "animal-side" blood-based test for antemortem detection of *M. bovis* is particularly appealing for TB surveillance programs of nontraditional livestock and wildlife. In vitro assays of cell-mediated immunity, such as the gamma interferon enzyme-linked immunosorbent assay (ELISA), have shown promise in this regard for *Mycobacterium tuberculosis* and *M. bovis* infection of humans (6) and cattle (36), respectively. They remain, however, complex tests that require processing of the blood sample within 24 h, are subject to complications associated with overnight delivery (e.g., temperature fluctuations and delays) (46), and involve an antigen stimulation step necessitating at least one working day to complete the test. Serological assays, in contrast, are rapid, inexpensive, easy to perform, and not subject to the sampling and processing variables of cellular assays, making them well suited to field sampling.

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To date, no assay detecting circulating antibody to *M. bovis* has shown adequate sensitivity or specificity suitable for standalone routine diagnostic use. Early attempts to apply serological assays to diagnosis of TB were hampered by considerable technical difficulties related to the choice of antigens, the immunoassay format, the phase of the infection, and the antibody isotype involved in the response. These initial assays used highly cross-reactive antigen preparations of *M. bovis*, such as crude cell sonicate (10), culture filtrate (34), purified protein derivative (PPD) from heat-killed cultures (12), and lipoarabinomannan (LAM) (40). In general, a lack of specificity was reported when these antigens were used, and the distribution of antibody titers between infected and noninfected individuals was widely overlapping. Subsequent attempts at using TB complex-specific antigens increased specificity but provided detection of serological responses in only a minority of infected animals (13). More recently, several additional protein antigens have been serologically characterized and display immunological specificity to *M. bovis* or the *M. tuberculosis* complex (27, 28). With such information, the use of antigen cocktails for TB diagnosis has shown promise in improving sensitivity (25) while maintaining high specificity (2). Studies to date, however, have shown variable animal-to-animal and species-to-species antigen recognition patterns (27, 45). As a result, the development of serological TB assays for wildlife species will require specific information about the antigens recognized by antibodies that are produced during *M. bovis* infection.

This report characterizes the antibody response of red deer-elk (*Cervus elaphus*) hybrid *Cervidae* to experimental infection with *M. bovis.* Several immunoassays were used to establish the kinetics and nature of reactivity of serum antibodies to crude mycobacterial antigens and a panel of highly purified recombinant proteins of *M. bovis*/*M. tuberculosis.* The effect of tuberculin skin testing on antibody response was also examined. Finally, the abilities of two serological rapid tests to correctly identify *M. bovis*-infected animals over the course of experimental infection were evaluated. The first, a lateral-flow immunoassay (CervidTB STAT-PAK), detects antibody reactivity to a mixture of target antigens (11, 26). This protocol was compared to a fluorescence polarization assay (FPA) specific for an MPB70 epitope previously developed for the detection of *M. bovis* antibodies in the sera of elk, bison, cattle, and llamas (41).

MATERIALS AND METHODS

Animals and infection. Fifteen healthy red deer (*Cervus elaphus elaphus*)-elk (*Cervus elaphus nelsoni*) hybrids of approximately 4 weeks of age were weaned and obtained from a herd with no history of TB and moved to the Ottawa Laboratory Fallowfield (OLF), Canadian Food Inspection Agency (CFIA), Ottawa, Ontario, Canada. Prior to the infection study, an outline of all procedures was approved by a CFIA Institutional Animal Care and Use Committee and was confirmed to be in accordance with the humane animal treatment requirements of the Canadian Council on Animal Care. At 8 months of age, 2 months prior to the experimental infection, animals were screened using a lymphocyte proliferation assay (15) against *M. bovis*, *M. avium*, and *M. avium* subsp. *paratuberculosis* tuberculins (PPD-bovis, PPD-avium, and PPD-Johnin, respectively; Biologics Production Unit, CFIA, Ottawa, Ontario, Canada) to confirm their TB-free statuses. For the duration of the study, 10 *M. bovis*-inoculated animals were housed in temperature- and humidity-controlled rooms (two or three animals per room) within a biosafety level 3 confinement facility. For the same duration, five control animals were housed within the same biosafety level 3 confinement facility and were treated similarly. Prior to inoculation, all animals were allowed to acclimate to their new environment for 2 weeks. Ten experimental red deer-elk hybrids were inoculated with a field strain of *M. bovis* (02/1007 [CFIA designation]) originally isolated from a beef cow in the Grandview, Manitoba area located adjacent to Riding Mountain National Park and possessing a spoligotype strain pattern identical to that reported for TB agent-infected wild elk from within the park (22). The challenge inoculum consisted of 1.5×10^3 CFU of mid-log-phase *M. bovis* grown in Middlebrook 7H9 media supplemented with 10% oleic acid-albumin-dextrose-citrate (Becton Dickinson, Mississauga, Ontario, Canada). To harvest tubercle bacilli from culture media, cells were pelleted by centrifugation at $750 \times g$, washed twice with 1 ml of phosphate-buffered saline (PBS; pH 7.2), and resuspended in PBS to an appropriate cell density for inoculation of 2 ml. After resuspension in PBS solution, serial 10-fold dilutions were inoculated on Middlebrook 7H10 agar slants supplemented with oleic acid-albumin-dextrose-citrate to confirm retrospectively the number of CFU administered. The challenge inoculum was instilled directly into the tonsillar crypts of anesthetized animals as previously described (33). Control cervids were treated similarly except that normal saline was instilled into tonsillar crypts. TB agent infection status was confirmed for all animals by careful postmortem examination and tissue sampling followed by mycobacterial culture and histopathology for acid-fast bacilli. All inoculated animals were positively infected, as *M. bovis* was isolated from sampled tissues and microscopic lesions consistent with TB contained acid-fast bacilli. Control animals remained uninfected, as tuberculous lesions were not identified, nor was *M. bovis* isolated. A detailed description of these methods has previously been described (32), and the results, including those for molecular testing by the amplified *M. tuberculosis* direct test (Gen-Probe, Inc., San Diego, CA) and spoligotyping (16), confirming that the *M. bovis* isolates were identical to the original inoculum, will be presented in a later publication.

Tuberculin skin testing. At 184 days postinoculation (p.i.), the in vivo delayed-type hypersensitivity reactions of control and inoculated animals to mycobacterial antigens were measured by the comparative cervical intradermal skin test (CCT). Briefly, hair was clipped from two sites on one side of the mid-cervical region, and the skin thickness of each site was measured. In the uppermost site, 0.1 ml of PPD-avium (0.5 mg/ml) was injected intradermally and 0.1 ml of PPD-bovis (1 mg/ml) was injected into the lower site. Tuberculins for skin test procedures were obtained from the Biologics Production Unit at the CFIA, Ottawa, Ontario, Canada. Injection sites were observed, palpated, and measured (Digimatic calipers; Mitutoyo Corporation, Tokyo, Japan) 72 h after injection. All measurements were performed in triplicate, and the average preinjection skin thickness (mm) readings were subtracted from the posttest readings to give values for both PPD-bovis and PPD-avium. Animals were categorized as negative, suspect, or reactor by plotting measurements on a scattergram developed by the CFIA for interpretation of the CCT for *Cervidae*.

ELISAs. Serological responses to LAM and PPD-bovis were evaluated by ELISAs in two laboratories, the National Animal Disease Center (NADC; USDA, Ames, IA) and the OLF (CFIA, Ottawa, ON, Canada) for LAM and PPD-bovis responses, respectively. These evaluations, described below, differed in terms of reagents (antigens and conjugates), test methodologies, and manners in which results were reported.

The LAM ELISA performed at the NADC involved the preparation of LAM-enriched antigens from *M. bovis* strain 95-1315 at the NADC as previously described (47). Immunlon II 96-well microtiter plates (Dynatech, Chantilly, VA) were coated with 100 μl/well (4 μg) of LAM-enriched antigen diluted with 0.01 M carbonate buffer (pH 9.6) and allowed to stand overnight at 4°C. Plates were washed three times with 200 μl/well PBS containing 0.05% Tween 20 (PBST; Sigma) and blocked with 200 μl/well commercial milk diluent/blocking solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After incubation for 1 h at 37°C in the blocking solutions, wells were washed nine times with 200 μl/ml PBST and test sera were added to the wells (100 μl/well). Test and control sera were diluted 1:100 in PBS containing 0.1% gelatin. Optimal dilutions of test sera were determined by evaluation of the reactivity of twofold serial dilutions ranging from 1:6 to 1:800 (volume of sera/volume of diluent ratio) with each of the antigens (47). After incubation for 20 h at 4°C with diluted test sera, the wells were washed nine times with 200 μl/well PBST and incubated for 1 h at 37°C with 100 μl/well horseradish peroxidase-conjugated polyclonal anti-cervine immunoglobulin G (IgG; heavy and light chains; Kirkegaard and Perry Laboratories) diluted 1:500 in PBS plus 0.1% gelatin. The wells were washed nine times with 200 μl/well PBST and incubated for 4.5 min at room temperature with 100 μl/well SureBlue 3,3′5,5′-tetramethyl benzidine (TMB) substrate (Kirkegaard and Perry Laboratories). The reaction was stopped by the addition of 100 μl/well 0.18 M sulfuric acid, and the A_{450}s of individual wells were measured with an automated ELISA plate reader (Molecular Devices, Menlo Park, CA). The optical density (OD) changes (ΔOD) were calculated by subtracting the mean

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OD readings for wells receiving coating buffer alone (two replicates) from the mean OD readings for antigen-coated wells (two replicates) receiving the same serum sample.

The PPD-bovis ELISA performed at the OLF used a trichloroacetic acid precipitate of PPD-bovis antigen prepared by the Biologics Production Unit (CFIA, Ottawa, Ontario, Canada). Round-bottom 96-well polystyrene plates (Nunc Maxisorb, Roskilde, Denmark) were coated with PPD-bovis at 1 μg/ml in 0.05 M carbonate buffer, pH 9.6. Immediately after the antigen was dispensed, the plates were frozen at −20°C and stored until used. All steps following antigen coating were conducted at room temperature. The wash and conjugate buffer was 0.01 M Tris, 0.15 M NaCl, pH 8.0, containing 0.05% Tween 20 and was used for sample dilution with the addition of 0.02% NaN_3. The test conditions consisted of overnight serum incubation with test samples diluted to 1:1,000 (100 μl/well). Washing of test samples was followed by 6 h of incubation with a conjugate (an alkaline phosphatase-labeled recombinant G protein [Zymed Laboratories, Inc., South San Francisco, CA]). The substrate *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) was added to diethanolamine buffer 20 min before use and the solution mixed well prior to use. Each plate was read in a kinetic assay with a microplate reader (Thermomax; Molecular Devices Corporation, Sunnyvale, CA), and any between-plate variation was corrected for using standards included on each plate. The test results are presented as slope values (milli-OD values/min) determined by using SOFTmax PRO software (Molecular Devices Corporation).

Electrophoresis and immunoblot assay. The antibody responses of cervids were evaluated over time by electrophoresis and immunoblot analysis using procedures described previously (1), with the following modifications. The antigens used for immunoblot analysis included a whole-cell sonicate (WCS) of *M. bovis* strain 95-1315 prepared as previously described (44) and recombinant MPB83 protein (Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, Northern Ireland). The antigen was electrophoresed through preparative 12% (wt/vol) polyacrylamide gels. Electrophoretic transfer of proteins onto pure nitrocellulose was accomplished with a Bio-Rad Trans Blot cell (Bio-Rad Laboratories, Mississauga, ON, Canada), using sodium phosphate buffer (25 mM, pH 7.8) at 0.8 A for 90 min. After transfer, the filters were blocked with PBST and 2% (wt/vol) bovine serum albumin (PBST-BSA). After being blocked, the filters were placed into a 20-slot mini-protean multiscreen device (Bio-Rad) and individual sera diluted 1:200 in PBST-BSA were added to independent slots. After 2 h of incubation at room temperature with gentle rocking, the blots were washed three times with PBST and incubated with horseradish peroxidase-conjugated polyclonal anti-cervine IgG (heavy and light chains; Kirkegaard and Perry Laboratories) diluted 1:20,000 in PBST-BSA for 1.5 h. The blots were again washed three times with PBST and developed for chemiluminescence in SuperSignal detection reagent (Pierce Chemical Co.).

Multiantigen print immunoassay (MAPIA). The following recombinant antigens of *M. tuberculosis*/*M. bovis* were purified to near homogeneity as polyhistidine-tagged proteins (designated by Rv numbers according to the classification of Cole et al. [4]): ESAT-6 (Rv3875) and CFP10 (Rv3874), produced at the Statens Serum Institut, Copenhagen, Denmark, and MPB59 (Rv1886c; a component of the Ag85 complex), MPB64 (Rv1980c), MPB70 (Rv2875), and MPB83 (Rv2873), produced at the Veterinary Sciences Division (Agri-Food and Biosciences Institute, Belfast, Northern Ireland). The 16-kDa alpha-crystalline protein (Acr1; Rv2031c) and the 38-kDa protein (PstS1; Rv0934) were purchased from Standard Diagnostics, Seoul, South Korea. Polyprotein fusions of ESAT-6/CFP10 and Acr1/MPB83 were constructed at the Statens Serum Institut by overlapping PCR using gene-specific oligonucleotides to amplify the genes from *M. tuberculosis* H37Rv chromosomal DNA. *Mycobacterium bovis* culture filtrate protein (MBCF) was obtained from a field strain of *M. bovis* (T/91/1378 [Veterinary Sciences Division]; cultured in synthetic Sauton's medium).

The MAPIA was performed as described previously (28), with the following modifications. Purified antigens were immobilized at a protein concentration of 0.05 mg/ml on strips of nitrocellulose membrane (Schleicher & Schuell, Keene, NH) to generate invisible parallel bands. Strips were blocked for 1 h with 1% nonfat skim milk in PBST and then incubated for 1 h at room temperature with individual serum samples diluted 1:50 in blocking solution. After being washed, the strips were incubated for 1 h at room temperature with peroxidase-conjugated protein G (Sigma) diluted 1:1,000 (Kirkegaard and Perry Laboratories), followed by another washing step. Cervid IgG antibodies bound to immobilized antigens were visualized with TMB (Kirkegaard and Perry Laboratories). The MAPIA results were scored by two independent operators, with a band of any intensity being read as a positive reaction.

Lateral-flow rapid test. A rapid immunochromatographic assay (CervidTB STAT-PAK test; Chembio Diagnostic Systems, Inc., Medford, NY) was evaluated for its ability to detect antibodies from experimentally inoculated animals. The test employed a cocktail of selected *M. tuberculosis* and/or *M. bovis* antigens, including ESAT-6, CFP-10, and MPB83, with a blue latex bead-based signal detection system (26). The test was performed according to the manufacturer's recommended procedure, using 30 μl of a serum sample and 3 drops of diluent buffer (included with the kit) that were added sequentially. The results were read visually 20 min after addition of diluent. Any visible band in the test area was considered indicative of an antibody-positive result. The absence of a band in the test area was considered an antibody-negative result.

FPA rapid test. The FPA was performed as previously described (41), with some modifications. Briefly, the serum was diluted 1:5 (total volume 1.0 ml) in PBS (0.01 M phosphate plus 0.85% sodium chloride, pH 7.4) supplemented with sodium azide (0.1%) and lithium dodecyl sulfate (0.1%) in an 11- by 75-mm borosilicate glass test tube. The mixture was vortexed and allowed to equilibrate for 2 h at room temperature, after which a blank reading was obtained with a fluorescence polarization analyzer (Sentry model; Diachemix LLC, Grayslake, IL). An aliquot of fluorescein-labeled MPB70 protein (sufficient to yield a total fluorescence intensity between 300,000 and 400,000 millipolarization [mP] values) was then added and the mixture vortexed. After equilibration for 30 min at room temperature, the blank-subtracted fluorescence mP value was measured. Sera from known infected and uninfected elk were included as positive and negative controls, respectively, in each batch of sera tested. All sera were tested in duplicate. Based on previous studies with elk sera, a delta mP value (defined as the mean mP value of the sample minus the mean mP value of the negative control) of ≥10 was scored as positive. A delta mP value of <10 was scored as negative.

Statistical analysis. ELISA data were analyzed by independent (infected versus noninfected) and paired (pre- versus post-skin testing) *t* tests, using a commercially available statistics program (MedCalc version 8.1.0.0; MedCalc Software, Mariakerke, Belgium). A *P* value of <0.05 was considered significant.

RESULTS

Serological response to crude mycobacterial antigens. Evaluation by ELISA of sequential serum specimens collected over the course of the infection study revealed significant ($P < 0.05$) antibody responses to *M. bovis*-derived LAM as early as 56 days p.i. (Fig. 1A). Antibody levels remained greater than those in control animals for the remainder of the infection study. A significant boost in antibody was seen following administration of tuberculin skin testing at 184 days p.i. ($P < 0.05$). The mean (± standard error) ELISA OD responses of infected animals increased from 0.86 ± 0.26 prior to skin testing (171 days p.i.) to 1.82 ± 0.19 1 week after skin testing (192 days p.i.). The time courses of antibody responses for most individual animals were characterized by multipeak kinetics (data not shown). Levels of serum antibodies to bovine tuberculin (PPD-bovis) were also detected by ELISA but, in this case, were not significantly different from those in control animals until after skin testing (Fig. 1B). Three of the ten *M. bovis*-inoculated cervids had no observable IgG responses to PPD-bovis throughout the infection study (data not shown).

Immunoblotting of *M. bovis* WCS with the sera of infected cervids demonstrated variable initial responses that were observed as early as 44 days p.i. for some animals. Specific bands of reactivity at 20, ~25, ~33, ~42, and ~75 kDa were consistently observed, with additional bands occasionally detected at <20 and >100 kDa (Fig. 2A). Responses were boosted both in intensity and in number of bands by injection of PPDs for the CCT. Responses to WCS prior to *M. bovis* infection and during the infection study for some noninfected cervids demonstrated the cross-reactivity to the crude antigen mixture (data not shown).

Serological responses to purified proteins. MAPIA performed on sequentially collected serum samples revealed variable individual animal antigen recognition patterns with respect to

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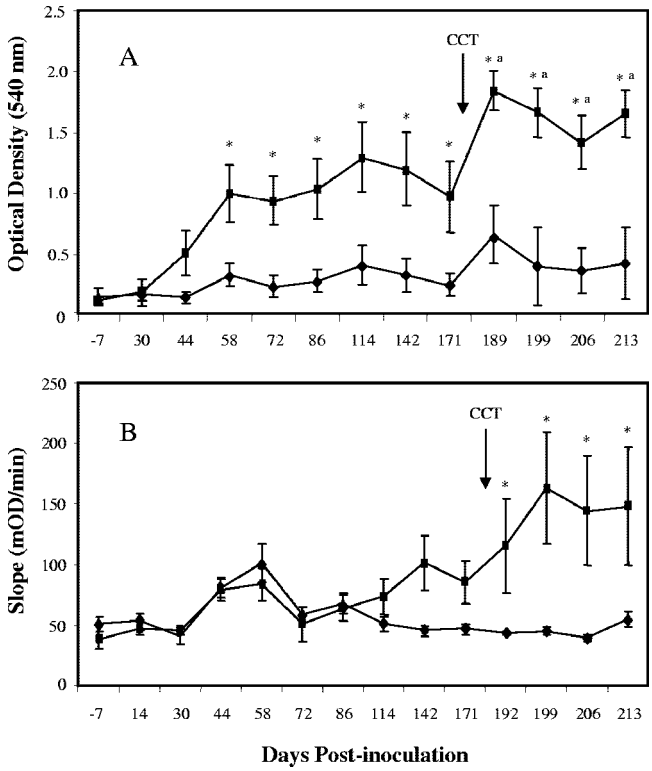

FIG. 1. Response kinetics of serum antibodies specific for *M. bovis* LAM-enriched antigen and PPD-bovis. Sera from *M. bovis*-inoculated (■, $n = 10$) and noninoculated (◆, $n = 5$) elk-red deer hybrids (*Cervus elaphus*) were analyzed for reactivity to *M. bovis*-derived LAM (A) and PPD-bovis (B) by ELISA. Results are presented as mean (± standard error of the mean) ΔOD readings (i.e., LAM response minus no antigen response) (A) and mean (± standard error of the mean) slope values (milli-OD values [mOD]) as measured by kinetic ELISA (B). * indicates antibody responses to LAM or PPD-bovis antigen that differ ($P < 0.05$) from the levels for control animals, and "a" indicates antibody responses of infected animals that differ ($P < 0.05$) from the levels observed immediately prior to administration of tuberculin for CCT at 184 days p.i.

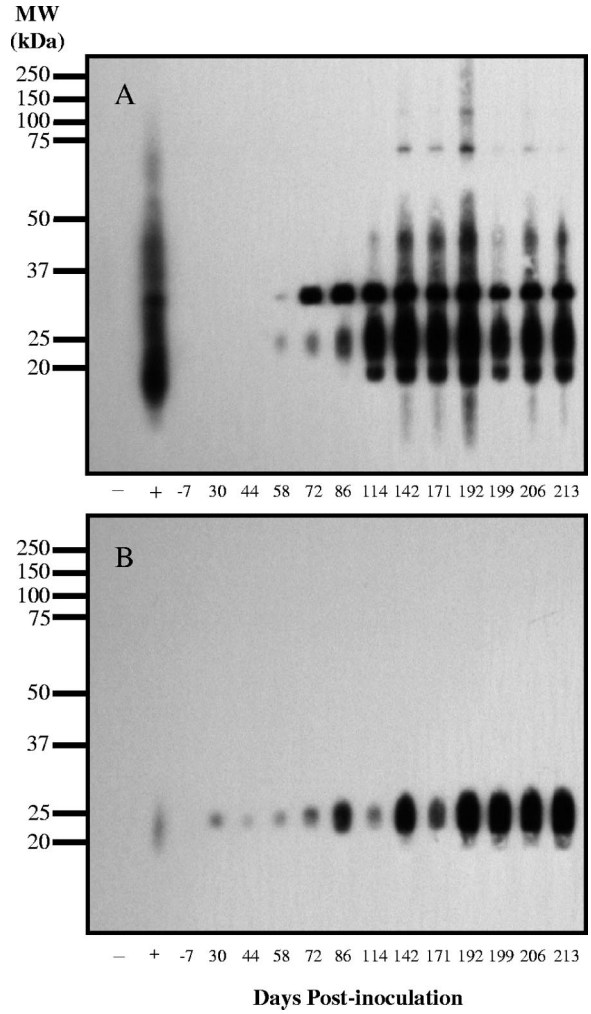

FIG. 2. Immunoblot of *M. bovis* 93-1315 WCS (A) and MPB83 (B) antigen probed with sera from cervids experimentally infected with *M. bovis*. The data presented are from a representative elk-red deer hybrid (*Cervus elaphus*) experimentally infected with 1.5×10^3 CFU of *M. bovis*. Molecular mass (MW) markers are indicated in the left margin, and numbers of days p.i. are indicated in the lower margin. CCT was performed at 184 days p.i.

both response kinetics and spectrum of antigen reactivity. Examples of MAPIA strips collected from infected cervids representing different antigen reactivity patterns are shown in Fig. 3, and the results for all animals are summarized in Table 1. Infected animals produced IgG antibodies starting at variable time points p.i., but for several animals, IgG antibodies were noted as early as 44 days p.i. Animal no. 36 showed strong and early (44 days p.i.) serum reactivity to ESAT-6, CFP10 and the corresponding hybrid fusion protein (Fig. 3). In contrast, animal no. 37 showed no antibody response to these antigens but reacted early (44 days p.i.) and consistently to MPB83. For some animals, there was a change in antigen immunodominance pattern over the infection period. For example, antibody from the serum of animal no. 44 transiently recognized MPB83 at days 44, 56, 72, and 86 p.i. A few weeks later, antibody response to MPB83 declined, whereas antibodies to ESAT-6, CFP10, and the corresponding fusion protein increased and became further pronounced post-skin testing.

Despite the variability, MPB83 was clearly the serodominant antigen, being recognized in 9 of 10 and 10 of 10 *M. bovis*-inoculated animals pre- and post-skin testing, respectively (Table 2). Antibodies to MPB83 were detected in some animals as early as 44 days p.i. by MAPIA, compared to 30 days p.i. for immunoblotting (Fig. 2B). For comparison, two complex mycobacterial antigens (PPD and MBCF) were included in the MAPIA and were recognized, at least at some time points, by 8 of 10 infected animals, which correlated with the PPD ELISA results. Notably, however, antibody to MBP83 was elicited prior to that of either PPD or MBCF (Fig. 3). Other antigens that were highly reactive by MAPIA included MPB70 (7/10 animals), CFP10 (7/10 animals), and ESAT-6 (8/10 animals) (Table 1). Only a few reactors against the MPB64, MPB59, and 38-kDa proteins were found, and the Acr1 protein elicited no antibody response. The Acr1/MPB83 fusion protein did not react with any more animals than did MPB83 alone. In contrast, the ESAT-6/CFP10 fusion protein reacted with 8 of 10 infected cervids prior to skin testing, demonstrating greater reactivity than either of the constituent proteins alone. Impor-

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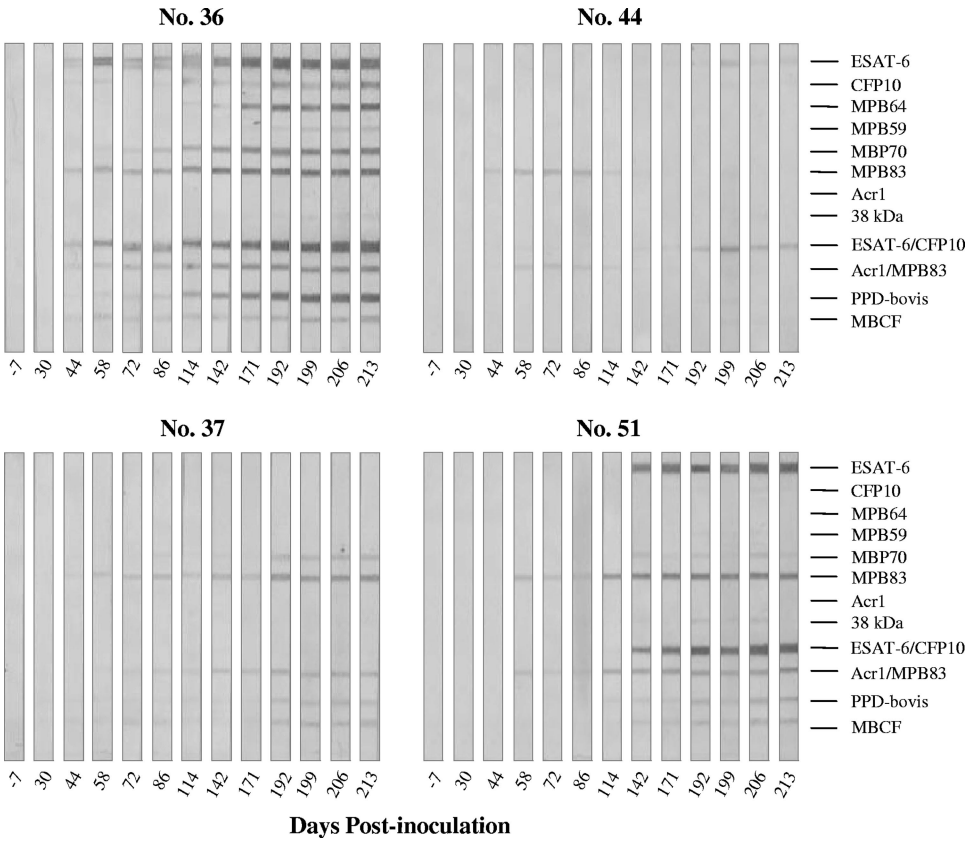

FIG. 3. Differential antibody responses of cervids experimentally infected with *M. bovis* to recombinant antigens detected by MAPIA. The responses of 4 of 10 *M. bovis*-infected animals (no. 36, 37, 44, and 51) are presented to illustrate the variability in antigen recognition patterns between animals. Each panel of strips shows results obtained with a set of sequential serum samples; each strip represents one serum sample collected at the time p.i. indicated. Bands on the MAPIA strips indicate the presence of antibodies to antigens printed onto a membrane (the antigens evaluated are listed on the right margin). CCT was performed at 184 days p.i.

tantly, a combination of all antigens detected all inoculated animals (10 of 10) at some point p.i. As was the case for the ELISA and immunoblotting experiments, injection of mycobacterial antigens at the time of intradermal tuberculin injection for skin testing boosted antibody responses to several antigens since MAPIA bands became more intense. Moreover, in some animals, additional bands appeared only after skin testing, showing tuberculin-induced antibodies to MPB83, ESAT-6, CFP10, MPB59, and MPB64. This resulted in an overall increase in the number of inoculated animals responding to particular antigens at all time points post-skin testing

TABLE 1. Variable antibody responses to protein antigens as detected by MAPIA for cervids experimentally infected with *M. bovis*

Antigen	Response[a] for indicated animal no.									
	36	37	40	41	44	48	51	116	118	119
ESAT-6	+	−	+	+	+	−	+	+	+	+
CFP10	+	−	+	+	+	−	+	+	+	−
MPB64	+	−	+	−	−	−	−	−	−	−
MPB59	+	−	+	+	+	−	−	−	−	−
MPB70	+	+	+	+	−	−	+	+	+	−
MPB83	+	+	+	+	+	+	+	+	+	+
Acr1	−	−	−	−	−	−	−	−	−	−
38-kDa protein	+	−	−	+	+	+	−	−	−	−
ESAT-6/CFP10	+	−	+	+	+	+	+	+	+	+
Acr1/MPB83	+	+	+	+	+	−	+	+	+	+
PPD-b	+	+	+	+	+	+	+	+	+	+
MBCF	+	+	+	+	+	+	+	+	+	+

[a] + and − indicate the presence and absence of circulating IgG antibodies over the 7-month infection period as detected by MAPIA for antigens of *M. bovis*/*M. tuberculosis*.

TABLE 2. Antigen recognition by serum antibodies of cervids infected with *M. bovis* as determined by MAPIA

Antigen	No. of antibody reactors[a] (n = 10)	
	Pre-skin testing	Post-skin testing
ESAT-6	7	8
CFP10	6	7
MPB64	2	2
MPB59	3	4
MPB70	6	7
MPB83	9	10
Acr1	0	0
38-kDa protein	3	4
CFP10/ESAT-6	8	9
Acr1/MPB83	7	9

[a] Values indicate the maximal rates of antigen recognition among infected animals as measured before and after CCT performed at 184 days p.i.

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TABLE 3. Summary of diagnostic serological responses as determined by different methods for sera of cervids (*C. elaphus*) experimentally infected with *M. bovis*

Test method	Rate (no. of animals with antigen/total no. of animals)[a] for indicated no. of days p.i.												Overall sensitivity (%)[b]
	30	44	58	72	86	114	140	171	192	199	206	213	
MAPIA	2/10	6/10	8/10	8/10	8/10	8/10	8/10	8/10	9/10	9/10	9/10	9/10	76.7
Lateral flow	2/10	5/10	9/10	7/10	7/10	7/10	7/10	8/10	8/10	9/10	9/10	9/10	72.5
FPA	0/10	0/10	0/10	0/10	3/10	4/10	6/10	5/10	6/10	6/10	5/10	5/10	33.3

[a] Values indicate rates of antigen recognition among infected animals as measured at various time points p.i. Tuberculin skin testing was performed at 184 days p.i.
[b] A composite diagnostic sensitivity based on all time points sampled over the infection study.

(Table 2). In particular, the numbers of responders to MPB83 and the ESAT-6/CFP10 fusion protein were 10 of 10 (100%) and 9 of 10 (90%), respectively, post-skin testing. The MAPIA responses were highly specific in that antibodies specific to *M. bovis* antigens were not found in the preinoculation sera or with the sera of uninfected control animals before or after skin testing (no false positives).

Evaluation of rapid antibody tests. The diagnostic performance levels of two rapid serodiagnostic tests were evaluated and compared to those for the MAPIA responses. The first, a lateral-flow test (CervidTB STAT-PAK), detected the antibody responses to multiple antigens, including MPB83, ESAT-6, and CFP10. These antigens were selected based on MAPIA results that, when combined, would be sufficient to cover animal-to-animal variation in antibody reactivity patterns. The second rapid test, an FPA, was developed to detect the antibody response to a single antigen (MPB70). Each test was evaluated with the same serum samples collected from the experimentally infected cervids over the course of the experimental infection. The abilities of the two rapid tests to detect *M. bovis*-infected animals were compared to the ability of the MAPIA at every test point (Table 3). In addition, a composite diagnostic sensitivity was determined, covering testing over the entire infection study. The lateral-flow test was able to detect serum antibody as early as 30 days after challenge, followed by consistent antibody detection in 7 to 8 of 10 infected animals over the following 23 weeks. The administration of tuberculin skin testing resulted in increased positivity, with 8 or 9 of 10 infected animals detected over the 4 weeks following the skin test. Overall, the lateral-flow test produced a composite infection study sensitivity of 73%. In contrast, the MPB70-specific FPA detected infected animals much later (86 days p.i.) did not exceed a sensitivity of 6 of 10 animals detected at any time during the infection study and had a composite infection study sensitivity of 33%. For comparison purposes, the MAPIA detected 2 of 10 infected animals as early as 30 days p.i., with 9 and 10 of 10 inoculated animals correctly identified as infected pre- and post-skin testing, respectively (Table 3). The overall infection study sensitivity for the MAPIA was 77%. The serological performance levels of the rapid tests were in agreement with the MAPIA results obtained on the same set of sera for the respective antigens. As for the MAPIA, both rapid test assays were highly specific in that at no time point were control animals incorrectly categorized as infected (no false positives).

DISCUSSION

This report characterizes the antigen recognition patterns to multiple mycobacterial antigens during experimental *M. bovis* infection of cervids (red deer-elk hybrids) and evaluates the utility of two rapid tests for the detection of infection. The kinetics of antibody response to LAM, a glycolipid present in large quantities on the surface of *M. bovis*, has been evaluated in studies of several species and was initially considered a useful candidate antigen for TB diagnosis (9, 40). Responses to the LAM-enriched antigen, however, were found to be broadly cross-reactive with nontuberculous mycobacteria and other closely related bacterial species. This effect was observed in the present study, where antibody to LAM was detected in some animals prior to *M. bovis* infection and in some control animals over the course of the study. For TB diagnostic applications, this cross-reactivity highlights the need for more-specific serological antigens for identification of *M. bovis*-infected animals, as it is likely that many captive and free-ranging cervids will have been exposed to environmental nontuberculous mycobacteria.

The use of individual purified proteins unique to *M. bovis* can improve the specificity of antibody-based TB tests but comes at the expense of diagnostic sensitivity. Employing multiple protein antigens covering a broad antigenic repertoire has shown promise in overcoming this limitation. The MAPIA is a powerful tool in identifying the most-frequently recognized antigens over the course of experimental *M. bovis* infection for a given host species. The result of this study was the identification of seroreactive targets not previously reported for red deer or elk TB. Variable animal-to-animal and temporal antigen recognition patterns consistent with previous observations made in studies of tuberculous cattle (27), badgers (11), and white-tailed deer (45) were observed. Importantly, however, the complete panel of proteins reacted with sera from all infected animals. Despite this variability, the 25-kDa antigen (MPB83) either alone or as a fusion partner with the 16-kDa alpha-crystalline (Acr1) protein was clearly the serodominant antigen, eliciting responses in 9 and 10 of 10 cervids pre- and post-skin testing, respectively. This surface-associated antigen of *M. bovis* has also been shown to be serodominant for white-tailed deer (45), reindeer (44), and Eurasian badgers (11). Interestingly, the one animal (no. 48) that did not react to the Acr1/MPB8 fusion protein did produce a response to the single MPB83 recombinant. This may have been the result of epitope inaccessibility in the generation of the fusion protein. In the present study, MPB83 responses were detected early and generally increased over time after inoculation. In all cases, antibody to MPB83 was elicited prior to that of either PPD or MBCF, both of which are complex mycobacterial antigens.

Additional highly reactive antigens included ESAT-6 and CFP10, which are immunogenic low-molecular-mass proteins secreted by virulent *M. tuberculosis* and *M. bovis* (38, 39).

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These antigens are highly specific to the *M. tuberculosis* complex and thus have demonstrated potential to improve test specificity. In the present study, the ESAT-6/CFP10 fusion protein showed greater reactivity by MAPIA than did corresponding single proteins, demonstrating the potential of polyepitope fusions in developing a serodiagnositic test for TB. This phenomenon was previously reported in human (19), badger (11), and white-tailed deer (45) studies. The *M. tuberculosis* complex CFP10 and ESAT-6 antigens have been shown to form a 1:1 heterocomplex potentially displaying additional conformation epitopes (35). Interestingly, the high rate of seroreactivity to ESAT-6/CFP10 reported here contrasts with recent studies of white-tailed deer (45) and reindeer (44) that showed much lower levels of seroreactivity (~30%) for the same hybrid protein.

Several other antigens, including MPB59, MPB64, and the 38-kDa protein, showed lower seroreactivity (2 or 3 of 10 animals). Although the 38-kDa protein is the most potent B-cell antigen in human TB (23), only 30% seroreactivity was observed in this study. It was surprising and important to note that no tuberculous animals recognized the 16-kDa Acr1 protein, since previous studies with cattle, white-tailed deer, and reindeer have all demonstrated seroreactivity (27, 44, 45). These findings illustrate the need to determine antigen recognition patterns in response to *M. bovis* infection for each species of interest.

A scientific paradigm held for decades was the belief that host defense to TB was primarily a function of cell-mediated immunity but that antibody played little or no protective role. Consistent with this paradigm was the early observation that serodiagnostic methods, in contrast to cell-mediated response assays, were able to detect antibody responses only at the later stages of TB agent infection. It is now recognized, however, that the immune response to any particular microorganism is neither strictly cell mediated nor antibody mediated in nature but rather involves a spectrum of reactivity. Indeed, several recent studies have demonstrated early antibody responses with beneficial effects on various aspects of TB agent infection (3, 48). In the present study, antibodies to some antigens, including MPB83, ESAT-6, and CFP10, were detected at low levels as early as 4 weeks after experimental infection. This was as early as the cell-mediated immune responses measured as part of this study but reported elsewhere (14). In contrast, seroreactivity to other antigens, including MPB70, a secreted stable protein of *M. bovis* and active component of bovine tuberculin (13), was detected much later in the infection period (>86 days).

To control and eradicate bovine TB from free-ranging and captive wildlife, it would be advantageous to apply rapid tests to surveillance programs that are low in cost and can be performed outside the laboratory (i.e., "animal-side"). Although numerous attempts to develop a rapid serodiagnostic test for human TB have been disappointing, the potential of this approach for TB detection in animals has recently been demonstrated (11, 25, 26). The MAPIA data of the present study have implications for the application of such test methods to *C. elaphus*. The variable response kinetics between antigens and, for some animals, the changing antigen recognition patterns over time will require a high-sensitivity test to include a cocktail of carefully selected antigens covering the broadest spectrum of antibody reactivity in a population. This study suggests that such a cocktail should include at least MPB83, ESAT-6, and CFP10.

The evaluation of the serological rapid tests, each with potential for animal-side application, supported the above conclusions. Each test used a small amount of serum or plasma and provided results within minutes. The lateral-flow immunoassay detected reactivity to a mixture of antigens, including ESAT-6, CFP10, and MPB83, whereas the FPA utilized MPB70 as a single diagnostic antigen. The MPB70 protein is secreted from *M. bovis*, forms a major component of *M. bovis* culture filtrate, is an active component of PPD tuberculin (13, 30), and is a dominant B-cell target in tuberculous cattle (8). Importantly, the selected antigens for both assays are highly specific for the *M. tuberculosis* complex, thus providing the necessary basis for a highly specific TB serodiagnostic test. Indeed, this was supported by the lack of reactivity in control animals over the study period. Both rapid tests detected serum antibodies in infected animals over the course of the infection study. The multiantigen lateral-flow test started to detect infected animals as early as 30 days p.i. (Table 3) and was able to detect antibodies from all infected animals at some point over the infection period. In contrast, it was not until 86 days p.i. that the MPB70 FPA started to classify infected animals as reactors, and 3 of 10 animals did not show any reactivity toward MPB70. The late reactivity to the MPB70 antigen was consistent with the MAPIA results for the same sera. Over the course of the infection study, the lateral-flow assay compared favorably to the MAPIA, with composite infection study sensitivities of 73% and 77%, respectively. This contrasted with the low composite sensitivity of the FPA, at 33%. Previously, seroreactivity to MPB70 has been high for TB agent-infected cattle (27), and the FPA was demonstrated to have a high diagnostic sensitivity (93%) for detecting Canadian cattle naturally infected with *M. bovis* (41). The comparatively low diagnostic sensitivity for cervids noted in this study suggests that there are significant species differences with respect to seroreactivity to MPB70. This finding further illustrates the variability of antigen recognition patterns between species and the advantage of incorporating multiple seroreactive antigens for TB diagnosis.

Tuberculin skin testing at 184 days p.i. resulted in elevated antibody responses by ELISA, immunoblot analysis, and MAPIA and increased the rates of disease detection by the rapid tests during the 4-week testing period post-skin testing. Such anamnestic antibody responses to mycobacterial antigens were initially recognized in early bovine TB control programs (17) and have periodically been rediscovered over the years (13, 49). For cattle, the boosted immunoglobulin responses post-skin testing have been shown to be an anamnestic IgG1 response (21) and to involve a number of antigens that are presumably present in tuberculin (13, 24). This phenomenon has been similarly reported in studies of various other species, including white-tailed deer and reindeer (44, 45). If this enhanced sensitivity is also found to be diagnostically specific, the boosting of *M. bovis*-specific antibody responses by skin testing could be exploited to improve TB serodiagnosis of captive wildlife. For example, a highly specific serological TB test could be used following standard skin-testing procedures (i.e., as a complementary or confirmatory test), which would serve to decrease the number of animals slaughtered due to false-

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positive skin test reactions. This approach, however, would prove impractical for free-ranging wildlife, as it requires an additional capture and handling of each animal following skin testing to collect serum and read the skin test.

It is likely that the diagnostic performance of serological rapid tests can be further improved. This study evaluated only a select group of protein antigens secreted by *M. bovis.* Recently, a TB-antigen microarray was used to screen human TB patients for antibodies to 54 oligosaccharides, polysaccharides, and lipopolysaccharides, TB antigens achieving high discriminatory power (43). In addition, other testing formats, such as latex bead agglutination assays, have also shown promise in TB serodiagnostics (18). The inclusion of the optimal mix of proteins, fusion proteins, lipoproteins, and lipopolysaccharides to enhance sensitivity, taken with the continuing miniaturization of serological assay formats, would seem to hold promise for the future of animal-side TB diagnostics. Ultimately, the main usage of such tests may be to monitor the prevalence of disease in various wildlife populations, to screen animals prior to entry into disease-free herds, or to monitor the spread of infection in zoological parks. The infection model used in this study produced tuberculous lesions with no associated clinical signs and thus closely mimics the disease state in naturally infected animals without advanced disease. A question remains, however, as to how well this model reflects all aspects of the disease process and the range of responses present in a naturally infected free-ranging population. Additional studies with greater numbers of well-defined positive and negative samples from naturally exposed cervids are required to evaluate these and other emerging antibody-based diagnostic platforms. In addition, further work is required to extend the application to other wildlife species of interest.

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