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Mycobacterium bovis–infected white-tailed deer (*Odocoileus virginianus*): detection of immunoglobulin specific to crude mycobacterial antigens by ELISA

W. Ray Waters, Mitchell V. Palmer, Diana L. Whipple

Abstract. White-tailed deer (*Odocoileus virginianus*) have recently emerged as a source of *Mycobacterium bovis* infection for cattle within North America. The objective of this study was to evaluate the antibody response of *M. bovis*–infected deer to crude mycobacterial antigens. Deer were experimentally inoculated with *M. bovis* strain 1315 either by intratonsilar instillation or by exposure to *M. bovis*–infected (i.e., in contact) deer. To determine the time course of the response, including the effects of antigen administration for comparative cervical skin testing, serum was collected periodically and evaluated by enzyme-linked immunosorbent assay (ELISA) for immunoglobulin (i.e., IgG heavy and light chains) reactivity to mycobacterial antigens. The reactivity to *M. bovis* purified protein derivative (PPDb) exceeded ($P < 0.05$) the reactivity to *M. avium* PPD (PPDa) only after in vivo administration of PPDa and PPDb for comparative cervical testing of the infected deer. The mean immunoglobulin response, as measured by ELISA, of intratonsilar-inoculated deer to a proteinase K–digested whole-cell sonicate (WCS-PK) of *M. bovis* strain 1315 exceeded ($P < 0.05$) the mean of the prechallenge responses to this antigen at approximately 1 month after inoculation and throughout the remainder of the study (i.e., ~11 months). This response also exceeded ($P < 0.05$) that of the uninfected deer. Although this is encouraging, further studies are necessary to validate the use of the proteinase K–digested *M. bovis* antigens in the antibody-based assays of tuberculosis.

White-tailed deer (*Odocoileus virginianus*) are wildlife reservoirs of *Mycobacterium bovis* infection of cattle in the northeastern part of Michigan.[23] Although impractical for wild deer, an assay for the antemortem detection of *M. bovis*–infected captive deer would be feasible and also beneficial for the tuberculosis eradication program within the USA. Traditional antemortem tests of *M. bovis* infection of cattle rely on cellular immune responsiveness to crude *M. bovis* antigens.[31] As with cattle, tests of cellular immune reactivity may also prove reliable for detecting tuberculosis in white-tailed deer.[20,33] One of these tests, the comparative cervical test of delayed-type skin hypersensitivity, although reliable, requires handling the deer twice, once at the time of antigen administration and again 72 hours later for the evaluation of the response. A test requiring a single-handling event would minimize the risk of capture-associated injuries such as lacerations, fractures, and capture myopathy (to which white-tailed deer are particularly prone) and may prove more practical for tuberculosis detection of captive deer. A test of cellular immunity requiring a single blood sample that is based on detection of interferon-γ produced in response to *M. bovis* antigen stimulation,[24] although useful for red deer (*Cervus elaphus*), has not proven useful with the samples from white-tailed deer (*O. virginianus*) (Waters, Palmer, and Whipple, unpublished data). The antibodies for this enzyme-linked immunosorbent assay (ELISA) of interferon-γ were developed for use with the samples from red deer and evidently do not cross-react with the samples from white-tailed deer interferon-γ. Lymphocyte proliferation (e.g., blastogenesis or fluorescence-based assays of proliferation), although useful for research purposes and also requiring only a single blood collection, is generally considered too insensitive and cumbersome for diagnostic laboratories.[32,34] A composite blood test combining a lymphocyte blastogenesis assay with an antibody-based ELISA of reactivity to *M. bovis* PPDb, *M. avium* PPDa, and the *M. bovis*–specific protein, MPB70, is approved for use in Cervidae within the USA.[8,9] Thus, antibody-based assays of *M. bovis* infection of white-tailed deer are appealing because of both ease of sample collection and enhancement of sensitivity when used in combination with assays based on cellular activation. Few studies examining the humoral responses of white-tailed deer to *M. bovis* infection, however, have been performed.[17]

Recently, an ELISA based on detection of antibody specific for a lipoarabinomannan-enriched antigen proved superior to other licensed antibody-based assays in the diagnosis of bovine paratuberculosis.[12] The antigen preparation used for this study was purified by proteinase K digestion of a bacterial cell lysate of *M.*

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avium subsp. *paratuberculosis.* It is speculated that the enhanced efficacy of this lipid/carbohydrate–enriched antigen preparation resulted from selective binding of the lipid portion of the antigen preparation, leaving the more immunogenic carbohydrate portion of the lipooligosaccharide free to react with the antibody within the sample. Others have demonstrated that the highly purified lipoarabinomannan or arabinomannan also has the potential for use in the antibody-based assays of mycobacterial infection.[7,16,25–27] Inclusion of polysaccharide antigens (e.g., lipoarabinomannan or arabinomannan) with protein antigens in multiantigen assays may enhance the overall sensitivity of the test by allowing detection of antibodies directed at both nonproteinaceous and proteinaceous epitopes of the mycobacterium.[25,28,29]

The objective of the present study was to evaluate by ELISA the serum immunoglobulin response of tuberculous white-tailed deer for reactivity to crude *M. bovis* antigens, including a proteinase K–digested whole-cell sonicate (WCS-PK) antigen. The effects of antigen (i.e., PPDb and PPDa) administration for comparative cervical test as well as the temporal kinetics of the response were also evaluated.

Materials and methods

Experimental animals challenge inoculum, bacteriology, and necropsy. White-tailed deer (1–3 yr of age) were either raised within a tuberculosis-free herd at the National Animal Disease Center (NADC), Ames, Iowa, or obtained from farmed white-tailed deer herds with no history of tuberculosis. Seven castrated males and 5 nonpregnant females were experimentally infected with *M. bovis* (i.e., 8 by intratonsilar inoculation and 4 by in-contact exposure) as described[18,19,32] and 2 castrated males and 2 nonpregnant females served as uninfected controls. The strain of *M. bovis* used for the challenge inoculum (i.e., strain 1315, Ames designation) was isolated from a white-tailed deer in Michigan in 1994.[23] The challenge inoculum consisted of either 300 (n = 4) or 2×10^8 (n = 4) colony-forming units (cfu) of mid–log-phase *M. bovis* grown in Middlebrook 7H9 media supplemented with 10% oleic acid–albumin–dextrose complex[a] (OADC) plus 0.05% Tween 80.[b,2] To harvest tubercle bacilli from the culture media, cells were pelleted by centrifugation at $750 \times g$, washed twice with 1 ml of phosphate-buffered saline solution (PBS, 0.01 M, pH 7.2), and diluted to the appropriate cell density in 2 ml of PBS. Enumeration of bacilli was by serial dilution plate counting on Middlebrook 7H11 selective media.[c] For intratonsilar inoculation, deer were restrained and anesthetized with ketamine[d] (6 mg/kg) and xylazine[e] (2 mg/kg) given intramuscularly. Effects of xylazine were reversed by intravenous administration of 4 mg/kg tolazoline.[f] The challenge inoculum was instilled directly into the tonsilar crypts of the anesthetized deer. The infected deer were housed in pens (2–4 deer/pen) inside a biosecurity level 3. Twenty-one days after intratonsilar inoculation, 4 noninoculated castrated males (i.e., in-contact deer) were anesthetized as described and moved into the facility housing the intratonsilar-challenged deer (i.e., with the deer receiving 2×10^8 cfu of *M. bovis*). Two in-contact deer were housed with 2 inoculated deer in a pen of approximately 45.72 m^2. In each pen the deer shared a common source of water and feed. Pens were inside a biosecurity level 3 building with negative airflow exiting the building through high-efficiency particulate air (HEPA) filters. Directional airflow was such that air from the animal pens was pulled toward a central corridor and passed through HEPA filters before exiting the building. Airflow velocity was controlled to provide 10.4 air changes/hr. Deer were fed a pelleted ration and alfalfa hay.

Intratonsilar-inoculated deer were euthanized at 4 mo (i.e., deer receiving 2×10^8 cfu) or 11 mo (i.e., deer receiving 300 cfu) after instillation of *M. bovis* into their tonsilar crypts. In-contact deer were euthanized 6 mo after cohabitation with intratonsilar-inoculated deer. All deer were euthanized by intravenous injection of sodium pentobarbital.[d] Various tissues were collected for bacteriologic culture and microscopic examination. Detailed descriptions of cellular immune responses, bacteriologic culture, histopathology, and gross necropsy results are presented elsewhere.[19,20,32,33]

Comparative cervical test. Before the experiment and at 3 and 8 mo after inoculation, intratonsilar-inoculated and control deer were tested for in vivo cellular immune reactivity to PPDa[g] and PPDb[g] by the comparative cervical skin test as described.[20] In-contact deer were skin tested before and after 2 mo exposure to intratonsilar-inoculated deer. Results were used to categorize deer as negative, suspect, or reactor in relation to exposure to *M. bovis.*[31]

Antigens. Antigens used for ELISA and immunoblot assays included PPDa, PPDb, and 2 antigens prepared from *M. bovis* strain 1315 cultures: a WCS of the bacilli and a WCS-PK. The WCS antigen was prepared from 4-wk *M. bovis* strain 1315 cultures grown in Middlebrook 7H9 media supplemented with 10% OADC. Bacilli were pelleted, sonicated in PBS, and further disrupted with 0.1–0.15-mm glass beads[h] in a bead beater[h] and then placed on ice. The preparation was centrifuged and the supernatant harvested and filtered (0.22 μm). The WCS-PK antigen was prepared by digestion of the WCS in a 1-mg/ml proteinase K[i] solution (50 mM Tris, 1 mM $CaCl_2$ buffer, pH 8.0) for 1 hr at 50 C. WCS and WCS-PK antigens were also prepared from *M. bovis* strain 2100 (i.e., isolated from a Texas dairy herd and heterologous to the challenge strain), using the same methods as described for preparation of the WCS and the WCS-PK of strain 1315. Protein concentrations of the WCS and WCS-PK antigens were determined using a protein determination kit.[j] The WCS and WCS-PK antigens were stored at −20 C until needed for the assays.

Enzyme-linked immunosorbent assay. Immulon II 96-well microtiter plates[k] were coated with 100 μl/well antigen prepared in carbonate/bicarbonate coating buffer (pH −9.6). Optimal dilutions of antigens were determined by evaluation of the reactivity of 2-fold serial dilutions of each antigen (i.e., ranging from 1 to 40 μg/ml), with known positive serum samples from an *M. bovis*–infected deer by ELISA. Optimal concentrations determined by this method were 20 μg/ml for WCS-PK and 5 μg/ml for PPDa, PPDb, and WCS. Antigen-coated plates, including control wells containing

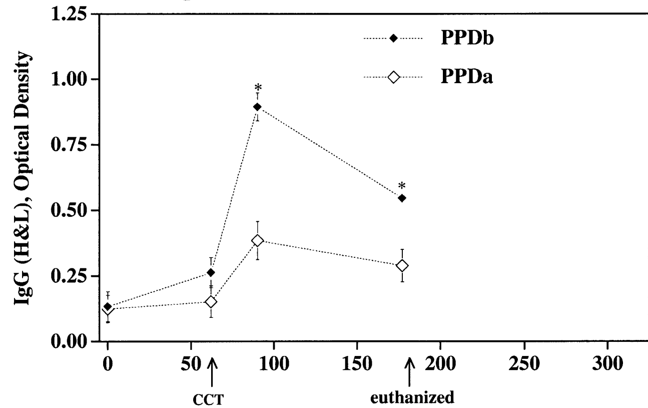
coating buffer alone, were incubated for 12–20 hr at 4 C. Plates were washed 3 times with 200 μl/well PBS containing 0.05% Tween 20[b] (PBST) and blocked with 200 μl/well of either PBS containing 1% gelatin, or a commercial milk diluent/blocking solution.[l] After incubation for 1 hr at 37 C in the blocking solution, wells were washed 3 times with 200 μl/well PBST, and test sera were added to the wells (100 μl/well). Test sera were diluted 1:100 in PBS containing 0.1% gelatin. Optimal dilutions of test sera were determined by evaluation of the reactivity of 2-fold serial dilutions ranging from 1:6 to 1:800 (volume sera:volume diluent, dilution with PBS containing 0.1% gelatin) with each of the antigens. After incubation for 1 hr at 37 C with the diluted test sera, the wells were washed 3 times with 200 μl/well PBST and incubated for 1 hr at 37 C with 100 μl/well of horseradish peroxidase–conjugated anticervine IgG heavy and light chains[l] diluted 1:1,000 in PBS plus 0.1% gelatin. The wells were washed 3 times with 200 μl/well PBST and incubated for 5–10 min at room temperature with 100 μl/well of 3,3′,5,5′-tetramethylbenzidine[l] (e.g., substrate). The reaction was stopped by addition of 100 μl/well of 0.18 M sulfuric acid[l] (stop solution), and the absorbances (450 nm) of individual wells were measured using an automated ELISA plate reader.[m] The change in optical density readings (i.e., Δoptical density) was calculated by subtracting the mean optical density readings for wells receiving coating buffer alone (2 replicates) from the mean optical density readings for antigen-coated wells (2 replicates) receiving the same serum sample.

Statistics. Mean Δoptical density readings were analyzed by one-way analysis of variance followed by Tukey–Kramer multiple comparisons test. Differences between groups were considered significant if probability values of $P < 0.05$ were obtained.

Results

Delayed-type hypersensitivity responses and infection status. All intratonsilar-inoculated deer were classified as reactors by the comparative cervical test 3 and 8 months after inoculation, and all in-contact deer were classified as reactors 2 months after exposure to intratonsilar-inoculated deer. All deer were classified as negative before exposure to *M. bovis.* Control deer were tested by the comparative cervical test at 3 time points corresponding to the same time points of testing for intratonsilar-inoculated deer. At each testing all control deer were classified as negative, with 1 exception. One control deer was classified as a suspect at the third time point (i.e., corresponding to the 8-month postinoculation skin test for the infected deer). All infected deer (i.e., intratonsilar-inoculated and in-contact exposed deer) had typical tuberculous lesions within their lungs, lung-associated lymph nodes, and/or head-associated lymph nodes upon necropsy.[20]

Enzyme-linked immunosorbent assay. Approved cellular-based assays of immunity to *M. bovis* infection rely on differential responses to crude mycobacterial extracts prepared from *M. avium* (PPDa) and *M. bovis* (PPDb) cultures. To determine both the temporal kinetics and the differential reactivity of serum from *M. bovis*–infected deer to PPDa and PPDb, sera from *M. bovis*–infected deer were collected periodically and evaluated by ELISA for antibody reactivity to these 2 antigens (Fig. 1). Mean Δoptical density readings of PPDb exceeded ($P < 0.05$) those of PPDa for the samples collected 1 month after administration of PPDb and PPDa for the comparative cervical test for both in-contact (Fig. 1A) and intratonsilar (Fig. 1B) exposed deer. Greater responses ($P < 0.05$) to PPDb as compared with responses to PPDa were also detected ~2 months after comparative cervical test for both in-contact exposed (Fig. 1A) and intratonsilar-inoculated deer (Fig. 1B, after the second test only). Reactivity



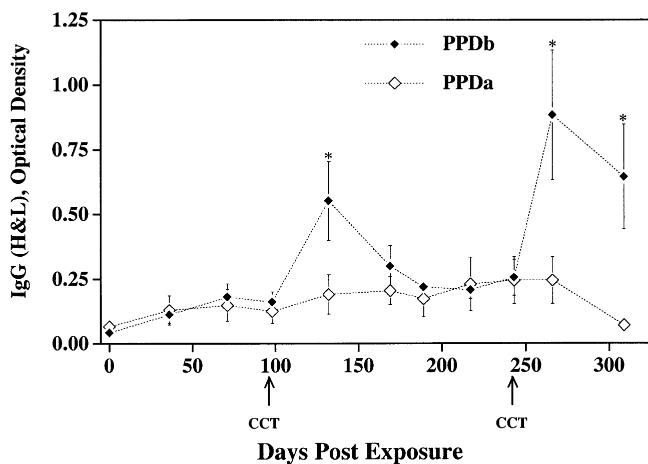
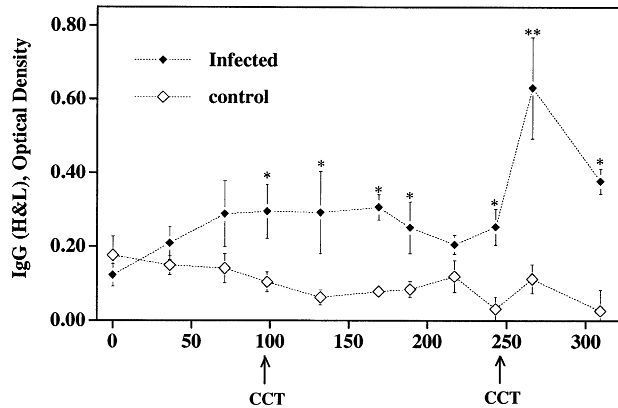


Figure 1. Serum ELISA of purified protein derivative (PPD) antigens derived from *M. avium* (PPDa) or *M. bovis* (PPDb). White-tailed deer were either exposed to experimentally infected deer (**A**, $n = 4$) or intratonsilarly inoculated with 300 cfu *M. bovis* (**B**, $n = 4$). CCT refers to a comparative cervical test (i.e., administration of PPDa and PPDb). Similar results were obtained from deer intratonsilarly inoculated with 2×10^8 cfu *M. bovis* (data not shown, $n = 4$). Results are presented as mean Δoptical density readings ± SEM. *, Exceeds ($P < 0.05$) the mean response to PPDa and the mean response of uninfected deer.

A. Whole Cell Sonicate



B. Proteinase k-Digested, Whole Cell Sonicate

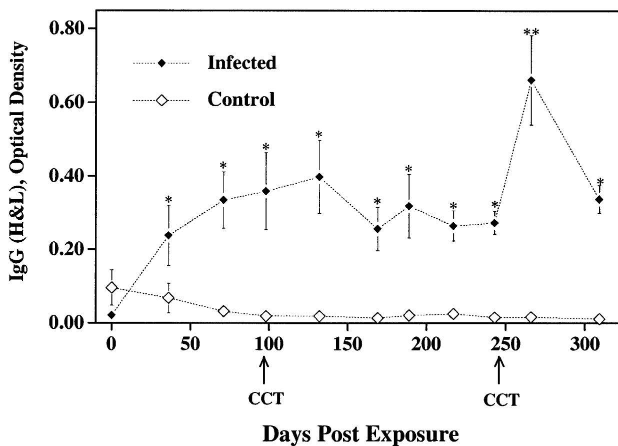


Figure 2. **A**, Serum ELISA of a WCS sonicate of *M. bovis,* and **B**, a proteinase K digest of the WCS antigen. White-tailed deer were intratonsilarly inoculated with 300 cfu *M. bovis.* CCT refers to a comparative cervical test (i.e., administration of PPDa and PPDb). Similar results were obtained using serum from deer exposed to experimentally infected deer (Table 1) or from deer intratonsilarly inoculated with 2×10^8 cfu *M. bovis* (data not shown, $n = 4$). Results are presented as mean Δoptical density readings ± SEM. *, Exceeds ($P < 0.05$) the mean response of uninfected deer. **, Exceeds ($P < 0.05$) the mean response of infected deer 240 days after exposure (i.e., immediately before the CCT).

to PPDb at each of these 5 time points (indicated by "*" in Fig. 1) was also higher ($P < 0.05$) than the responses to PPDb at the time of inoculation (i.e., prechallenge sera from the same deer) and the responses from control, uninfected deer collected at the same time points. Reactivity to PPDb at all other time points did not, however, exceed ($P < 0.05$) the response detected with sera from control deer collected at the same time points.

Reactivity of sera to WCS and WCS-PK antigens from intratonsilar-inoculated deer was compared with that of sera from uninfected deer (Fig. 2). Reactivity to the WCS antigen by sera from infected deer exceeded ($P < 0.05$) that of uninfected deer, beginning

Table 1. Comparison of serum immunoglobulin (IgG, heavy and light chains) responses of in-contact exposed deer to either *M. bovis* WCS or *M. bovis* WCS-PK antigen preparations.†

Days after exposure, status	WCS	WCS-PK
0, Prechallenge	0.246 ± 0.059	0.101 ± 0.070
62, Pre-CCT	0.353 ± 0.063	0.302 ± 0.092
90, Post-CCT	0.866 ± 0.068*	1.001 ± 0.095*
177, Necropsy	0.697 ± 0.107*	0.743 ± 0.128*

† Data are presented as mean ± SEM of Δoptical density readings for the response of sera from in-contact exposed deer to the respective antigens, $n = 4$.

* Exceeds ($P < 0.05$) the prechallenge values for the homologous antigen preparation (i.e., vertical comparisons). Differences between the two antigen preparations (i.e., horizontal comparisons) were not detected ($P > 0.05$).

3 months after inoculation and continuing throughout the study period except for 220 days after inoculation (Fig. 2A). A significant ($P < 0.05$) increase in reactivity to the WCS by infected deer was detected 1 month after the second administration of PPDs for the comparative cervical test (Fig. 2A). Likewise, a significant ($P < 0.05$) increase in the response to WCS-PK was also detected at the same time point (Fig. 2B). In addition, 1 month after intratonsilar challenge with *M. bovis* and at all time points throughout the study, the serum antibody response of infected deer to the WCS-PK exceeded ($P < 0.05$) that of sera from control deer to the WCS-PK (Fig. 2B). The response of infected deer to the WCS-PK was not statistically different (i.e., $P > 0.05$) from the response of the same deer to the WCS. Similar results were obtained with sera from in-contact exposed deer (Table 1). Although not statistically significant, prechallenge values for the WCS-PK were less than prechallenge values for the WCS antigen preparation (Table 1).

As inferred from the above results, the level of the response to each antigen was influenced by inherent or nonspecific reactivity to that particular antigen. To demonstrate the differences in this nonspecific reactivity, comparisons were made between the mean responses of sera collected from the uninfected deer ($n = 4$) to each antigen averaged throughout the study period (Table 2). The average response to WCS-PK was significantly ($P < 0.01$) less than the response to WCS, PPDa, or PPDb. Although the response by control deer to PPDa and PPDb did not differ, the response to the WCS was significantly ($P < 0.05$) less than the response to PPDb but not to PPDa.

Discussion

With the emergence of *M. bovis* infection in white-tailed deer, it has become necessary to develop tests to detect *M. bovis*–infected deer within captive herds.

Table 2. Comparison of immunoglobulin (IgG, heavy and light chains) responses of serum from uninfected deer to *M. bovis* antigens.†

Antigen	Mean ± SEM
PPDa	0.132 ± 0.014
PPDb	0.170 ± 0.016
WCS	0.099 ± 0.014*
WCS-PK	0.031 ± 0.008**

† Data are presented as mean ± SEM of Δoptical density readings for the response of 11 serum samples (diluted 1:100) collected monthly from 4 control deer to the respective antigens. Means were calculated by averaging the Δoptical density readings during the course of the study (i.e., 11 samples obtained monthly) to determine the average response to the respective antigen for each deer. Means for the 4 uninfected deer were then calculated from these average responses.

* Significantly ($P < 0.05$) less than the response to PPDb.

** Significantly ($P < 0.01$) less than the response to PPDa, PPDb, and WCS.

Antibody-based assays are particularly appealing for use with deer because the technology is easily transferable to diagnostic laboratory personnel, anticervine immunoglobulin detection antibodies are available, and sample collection requires a single-handling event, which minimizes injuries from capture. In addition, inclusion of serological assays in combination with tests relying on cellular activation may enhance detection of infected deer.[7,9,21] Findings from the present study demonstrate that detection of *M. bovis*–specific antibody responses is, indeed, feasible.

An important issue in the development of a diagnostic assay is the selection of antigen(s) for use in the test. Although complex mycobacterial antigens such as culture filtrates, WCS, and PPD are relatively inexpensive and easily produced, tests of reactivity to those complex antigens often lack sensitivity and specificity.[1,3,30] Conversely, the use of recombinant or native *M. bovis* proteins, such as MPB59, MPB64, MPB70, and MPB83, may enhance test specificity.[13–15] These proteins, however, are difficult to synthesize and/or purify and may lack sensitivity when used individually.[4,35] Assays of antibody reactivity to multiple recombinant proteins (i.e., cocktail based) should, without compromising specificity, increase the sensitivity of the test by enabling detection of diverse host responses.[5,6,14,15] It has been recently reported that certain recombinant mycobacterial proteins are poorly recognized in comparison with native proteins, and antibody generated by infection is likely more homogenous in reactivity than was previously presumed.[22] In preliminary studies, specific responses to recombinant *M. bovis* proteins (e.g., MPB70, MPB83) by sera from *M. bovis*–infected white-tailed deer are detectable (authors in collaboration with K. Lyashchenko [Chembio Diagnostic System, Medford, NY] and J. Pollock [Department of Agriculture for Northern Ireland, Stormont, Belfast, UK], data not shown). Further studies are ongoing to determine the utility of these recombinant proteins in antibody-based assays for the diagnosis of tuberculous deer.

As determined previously in studies with cattle and red deer,[9–11,13] administration of PPDs for skin testing boosts serum *M. bovis*–specific antibody responses of infected white-tailed deer. Thus, the enhanced antibody response detected 1 month after administration of PPDs for skin testing may be used for confirmation or clarification of the results obtained with the comparative cervical test. Further studies are necessary to fully characterize the kinetics of the antibody response boosted by PPD administration.

Not surprisingly, antigens processed from bacilli homologous to the challenge inoculum (i.e., WCS and WCS-PK) were more useful than nonhomologous antigen preparations (i.e., PPDb) in the detection of *M. bovis*–infected deer by ELISA. Unfortunately, sera from *M. bovis* strain 1315 infected deer did not react by ELISA with a WCS-PK prepared from a heterologous strain of *M. bovis,* strain 2100 (data not shown). The utility of this assay as a diagnostic tool will require cross-reactivity to other *M. bovis* isolates. Further studies are ongoing to characterize the reactivity of sera from *M. bovis*–infected deer as well as sera from deer infected with other mycobacteria to PPDs prepared from homologous and nonhomologous strains and species of mycobacteria.

In conclusion, it was demonstrated that white-tailed deer generate an antibody response to crude *M. bovis* antigens upon *M. bovis* infection. The response to experimental infection was detectable 1 month after inoculation when a simply prepared WCS-PK of *M. bovis* homologous to the challenge strain was used for coating the ELISA plates. Others have determined that this method of antigen preparation enriches for lipid and carbohydrate antigens (e.g., lipoarabinomannan). The application of these findings to field detection of tuberculous white-tailed deer is yet to be determined.

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a. Difco, Detroit, MI.
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f. Lloyd Laboratories, Shenandoah, IA.
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i. Roche Molecular Biochemicals, Indianapolis, IN.
j. Bio Rad, Hercules, CA.
k. Dynatech; Chantilly, Virginia, VA.
l. Kirkegaard and Perry Laboratories, Gaithersburg, MD.
m. Molecular Devices, Menlo Park, CA.

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