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Evaluation of an in vitro blood-based assay to detect production of interferon-γ by *Mycobacterium bovis*–infected white-tailed deer (*Odocoileus virginianus*)

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Abstract. Tuberculosis due to *Mycobacterium bovis* in captive Cervidae was identified as an important disease in the United States in 1990 and prompted the addition of captive Cervidae to the USDA Uniform Methods and Rules for eradication of bovine tuberculosis. As well, *M. bovis* infection was identified in free-ranging white-tailed deer in northeast Michigan in 1995. Tuberculosis in both captive and free-ranging Cervidae represents a serious challenge to the eradication of *M. bovis* infection from the United States. Currently, the only approved antemortem tests for tuberculosis in Cervidae are the intradermal tuberculin skin test and the blood tuberculosis test (BTB). At present, the BTB is not available in North America. Tuberculin skin testing of Cervidae is time-consuming and involves repeated animal handling and risk of injury to animals and humans. This study evaluated the potential of a new blood-based assay for tuberculosis in Cervidae that would decrease animal handling, stress, and losses due to injury. In addition, a blood-based assay could provide a more rapid diagnosis. Twenty 6–9-month-old white-tailed deer, male and female, were experimentally inoculated by instillation of 300 colony-forming units of *M. bovis* in the tonsillar crypts. Seven, age-matched uninfected deer served as controls. Blood was collected on days 90, 126, 158, 180, 210, 238, 263, and 307 after inoculation and was analyzed for the production of interferon-γ (IFN-γ) in response to incubation with *M. bovis* purified protein derivative (PPDb), *M. avium* PPDa, pokeweed mitogen (PWM), or media alone. Production of IFN-γ in response to PPDb was significantly greater ($P < 0.05$) at all time points in samples from *M. bovis*–infected deer as compared with uninfected control deer, whereas IFN-γ production to PWM did not differ significantly between infected and control deer. Measurement of IFN-γ production to PPDb may serve as a useful assay for the antemortem diagnosis of tuberculosis in Cervidae.

Although bovine tuberculosis has nearly been eradicated from the United States, the disease has persisted and the prevalence has increased in recent years. Factors that contributed to the resurgence of tuberculosis in animals are the importation of tuberculous cattle from Mexico, the persistence of low levels of *Mycobacterium bovis* infection in large dairy herds in the southwestern United States, and the presence of tuberculosis in captive deer and elk that are raised for agricultural purposes, in zoo animals, in game parks, in other exotic animal collections, and in free-ranging white-tailed deer.[15]

In the past 2 decades, there has been rapid expansion of the captive cervid industry in the United States. *Mycobacterium bovis* infection in captive cervids was identified as an important disease in the United States in 1990 when an outbreak of tuberculosis in captive elk in Canada was traced to elk imported from the United States.[2] Since then, tuberculosis has been diagnosed in at least 37 captive cervid herds in 18 states. This has prompted the addition of captive cervids to the USDA Uniform Methods and Rules for the eradication of bovine tuberculosis. As well, *M. bovis* infection was identified in free-ranging white-tailed deer in northeast Michigan in 1995.[11] Tuberculosis in both captive and free-ranging cervids represents a serious challenge to eradication of *M. bovis* from the United States. Currently, the only approved antemortem tests for tuberculosis in Cervidae are the intradermal tuberculin skin test that uses purified protein derivative (PPD) from *M. bovis* (PPDb) and *M. avium* (PPDa) and the blood tuberculosis test (BTB) that is a composite test measuring both cellular and humoral immune responses. At present, the BTB is not available in North America. Intradermal tuberculin testing in Cervidae lacks sensitivity and specificity;[1,4,5,8] moreover, animals cannot be retested for 90 days. Infection with nontuberculous mycobacteria often confounds the interpretation of skin testing, resulting in false-positive reactions. A blood-based assay for tuberculosis in cervids would decrease animal handling, stress, losses due to injury and allow short-interval repeat testing if necessary.

A whole-blood cellular assay using a sandwich en-

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Table 1. List of mitogens and concentrations used to stimulate whole blood from white-tailed deer for measurement of IFN-γ production.

Mitogen	Final concentration* (μg/ml)
Mycobacterium avium PPD (PPDa)	20
M. bovis PPD (PPDb)	20
Pokeweed Mitogen (PWM)	20
	10
	5
Concanavilin A	20
	10
	5
PHA	20
	10
	5
PBS	

* Concentration after addition of 1.5 ml of heparinized whole blood.

zyme immunoassay for bovine interferon-γ (IFN-γ) was previously developed and found to be effective in the diagnosis of *M. bovis* infection in cattle.[10,20] The IFN-γ immunoassay for cattle uses 2 monoclonal antibodies specific for biologically active bovine IFN-γ that do not detect bovine IFN-α or -β. The antibodies also recognize IFN-γ from sheep, goats, and buffalo but not from pigs, deer, or humans.[10] Development of monoclonal antibodies to cervid (*Cervus elaphus*) IFN-γ has resulted in an immunoassay for cervid IFN-γ similar to that in use for cattle.[12] Interferon-γ, a lymphokine, produced primarily by T-lymphocytes and natural killer cells, has been shown in animal models to be important in controlling infections by a number of intracellular pathogens including *M. tuberculosis.*[3] Stimulation of macrophages by IFN-γ is essential for effective killing of *M. tuberculosis,* which occurs primarily through the generation of nitric oxide and reactive nitrogen intermediates.[3]

The objectives of this study were to evaluate the ability of the cervid IFN-γ assay to detect IFN-γ from white-tailed deer (*Odocoileus virginianus*) and to determine its potential usefulness in detecting *M. bovis*–infected white-tailed deer using experimentally infected deer.

Materials and methods

Mitogen selection. To determine the best mitogen for use as a positive-control sample, blood was collected from 11 white-tailed deer, 1–1.5 yr of age, from a herd with no history of *M. bovis* infection. The whole-blood culture system for IFN-γ analysis has been described previously.[10,18] In brief, blood was collected in 10-ml vacuum tubes containing heparin as anticoagulant. A 1.5-ml aliquot of whole blood was added to separate 2-ml microcentrifuge tubes, each containing one of the mitogens listed in Table 1. Antigen was added to whole blood within 8 hr of collection. After adding PPDs,[a] mitogens,[b] or phosphate-buffered saline (PBS), tubes were capped, mixed by inversion or gentle vortexing, and incubated for 48 hr at 37 C in a humidified chamber with 5% CO_2. After incubation, samples were centrifuged, and plasma was collected and stored at −20 C until analyzed. Plasma samples were analyzed for the presence of cervid IFN-γ using a commercially available sandwich enzyme immunoassay kit[c] according to manufacturer's instructions. Optical density (OD) measurements were analyzed in duplicate at 450 nm using an automated microplate reader.

Experimental infection of deer. Twenty, 6–9-mo-old, white-tailed deer, male and female, were experimentally inoculated by instillation of 300 colony-forming units (CFU) of *M. bovis* in the tonsillar crypts as described previously.[7] Seven, age-matched noninoculated deer served as controls. For intratonsillar inoculation, deer were anesthetized by intramuscular injection of a combination of xylazine[d] (2 mg/kg) and ketamine[e] (6 mg/kg). After inoculation, the effects of xylazine were reversed by intravenous injection of tolazoline[f] (4 mg/kg). Blood was collected on days 90, 126, 158, 180, 210, 238, 263, and 307 after inoculation. For each deer, at each time point, 1.5 ml of blood was dispensed into separate 2-ml microcentrifuge tubes containing 0.1 ml each of PPDa (20 μg/ml final concentration), PPDb (20 μg/ml final concentration), pokeweed mitogen (PWM) (20 μg/ml final concentration), and PBS as described above. Samples were processed and analyzed as described above for the presence of cervid IFN-γ. Mean OD readings between groups and time points were compared using a Student's *t*-test. Differences were considered significant when $P < 0.05$.

Five deer each were euthanized with intravenous sodium pentobarbital and examined on days 90, 180, 263, and 307 after inoculation. Tissues were collected and processed as described previously[6] for bacteriologic culture and microscopic evaluation to confirm *M. bovis* infection.

Skin testing. Before inoculation, 96 and 235 days thereafter, delayed-type hypersensitivity (DTH) to *M. bovis* was measured by the comparative cervical intradermal skin test as described in USDA, APHIS, Veterinary Services guidelines.[13] Hair was clipped from 2 sites on 1 side of the midcervical region, and the skin thickness of each site was measured. One-tenth milliliter PPDa[g] (0.4 mg/ml) was injected intradermally in the uppermost site, and 0.1 ml of bovine PPDb[g] (1 mg/ml) was injected into the lower site. Injection sites were observed, palpated, and measured 72 hr after in-

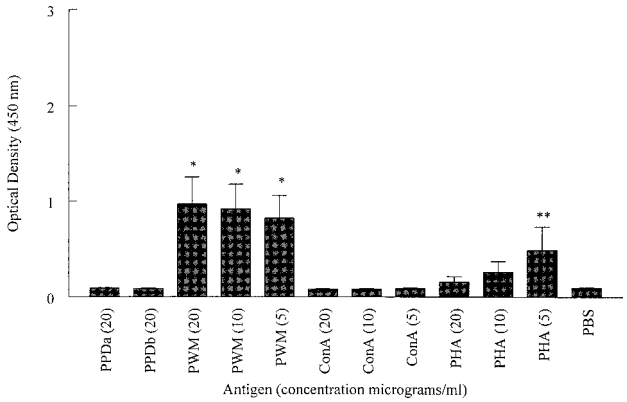

Figure 1. Interferon-γ production in response to stimulation with various mitogens at various concentrations as measured by OD (450 nm) using a commercial assay[c] for cervid IFN-γ. Data represent the mean response of 11 deer. *Differs from response to PBS alone, $P < 0.01$, **$P < 0.05$. PPDa = *M. avium* purified protein derivative, PPDb = *M. bovis* purified protein derivative, PWM = pokeweed mitogen, ConA = concanavilin A, PHA = phytohemagglutinin, PBS = phosphate buffered saline.

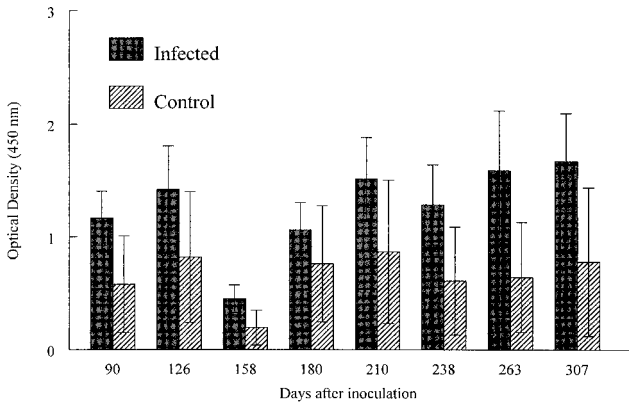

Figure 2. Interferon-γ production in response to stimulation with PWM as measured by OD (450 nm) using a commercial assay[c] for cervid IFN-γ. Infected deer were inoculated by intratonsillar instillation of 300 CFU of *M. bovis.* Control deer received no inoculation. Background OD values (stimulation with PBS alone) have been subtracted from the response to PWM. Control deer, $n = 7$ at all time points. Infected deer, $n = 20$ on day 90, $n = 15$ on days 126, 158, and 180, $n = 10$ on days 210, 238, and 263, $n = 5$ on day 307. The responses of infected and control deer at each time point did not differ ($P < 0.05$).

jection. Results were interpreted by plotting measurements on a graph (VS form 6-22D) developed by USDA for interpretation of the comparative cervical skin test for Cervidae. Based on the results, animals were categorized as negative, suspect, or reactor.

Results

Results of the mitogen selection phase of the study showed that IFN-γ production was greatest in response to stimulation with PWM (Fig. 1). Differences in IFN-γ production in response to the different concentrations of PWM used in this study were not significant; however, all responses to PWM, at all concentrations, were significantly greater than the response to PBS alone. Stimulation with phytohemagglutinin (PHA) at a concentration of 5 μg/ml was also significantly greater than stimulation with PBS alone. Because of the observed trend of increasing IFN-γ production with decreasing concentrations of PHA, further studies were carried out with PHA stimulation using concentrations of 1.0 and 0.1 μg/ml. These studies did not reveal stimulation greater than that seen with 5 μg/ml (data not shown). Stimulation with PPDa, PPDb, or concanavilin A, at any of the concentrations used in this study, did not result in IFN-γ production greater than that seen by stimulation with PBS alone. Based on these findings, for the remainder of the study, PPDa, PPDb, and PWM were all used at a concentration of 20 μg/ml.

Delayed-type hypersensitivity to *M. bovis*, as measured by comparative cervical skin testing, was not seen in any deer before inoculation. Ninety-six and 235 days after inoculation, all experimentally inoculated deer exhibited DTH consistent with exposure to *M. bovis* and were categorized as reactors. No negative-control deer developed a DTH response to *M. bovis* PPD. All experimentally inoculated deer developed tuberculosis, confirmed by bacteriologic culture and microscopic examination of multiple tissues. A detailed description of lesions has been reported elsewhere.[6]

In response to stimulation with PWM, both *M. bovis*–inoculated and noninoculated deer produced measurable IFN-γ at all time points (Fig. 2). Although production of IFN-γ in response to PWM was greater in *M. bovis*–inoculated deer than in noninoculated deer, the difference was not significant. In response to PPDd, IFN-γ production was greater ($P < 0.01$) in *M. bovis*–inoculated deer than in noninoculated deer (Fig. 3). Interferon-γ production in response to PPDa was significantly greater ($P < 0.05$) in inoculated deer than in noninoculated deer at 4 of 8 time points (Fig. 4). However, in *M. bovis*–inoculated deer, production of IFN-γ was always greater in response to PPDb than that seen in response to PPDa; the differences being significant ($P < 0.05$) at all points except 210 days after inoculation (Fig. 5).

According to the manufacturer of the Cervigam™ kit, PPDb-stimulated blood plasma from deer having an OD value greater than 0.050 above that of PPDa- and PBS-stimulated plasma indicates the presence of *M. bovis* or *M. tuberculosis* infection. Over the course of the current study, 44 tests were conducted on 7 different noninoculated deer. Of these, 43 (98%) were interpreted as negative, yielding 1 (2%) false-positive result from 1 deer at 1 time point. Ninety-one tests were conducted on 20 different *M. bovis*–inoculated deer. Sixty-seven

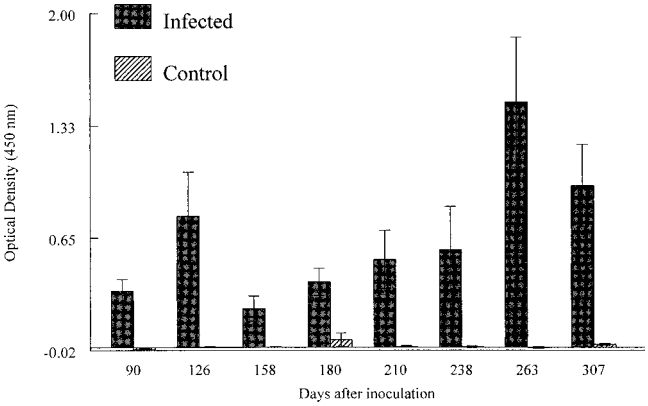

Figure 3. Interferon-γ production in response to stimulation with PPDb as measured by OD (450 nm) using a commercial assay[c] for cervid IFN-γ. Infected deer were inoculated by intratonsillar instillation of 300 CFU of *M. bovis.* Control deer received no inoculation. Background OD values (stimulation with PBS alone) have been subtracted from the response to PPDb. Control deer, $n = 7$ at all time points. Infected deer, $n = 20$ on day 90, $n = 15$ on days 126, 158, and 180, $n = 10$ on days 210, 238, and 263, $n = 5$ on day 307. The responses of infected and control deer at each point did not differ significantly ($P < 0.01$).

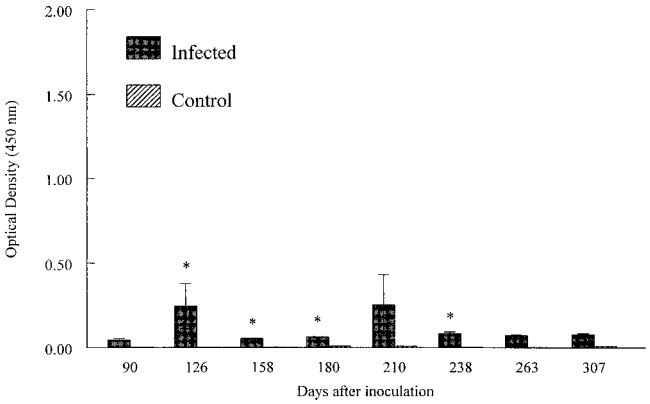

Figure 4. Interferon-γ production in response to stimulation with PPDa as measured by OD (450 nm) using a commercial assay[c] for cervid IFN-γ. Infected deer were inoculated by intratonsillar instillation of 300 CFU of *M. bovis.* Control deer received no inoculation. Background OD values (stimulation with PBS alone) have been subtracted from the response to PPDa. Control deer, $n = 7$ at all time points. Infected deer, $n = 20$ on day 90, $n = 15$ on days 126, 158, and 180, $n = 10$ on days 210, 238, and 263, $n = 5$ on day 307. *Responses by infected deer differ ($P < 0.05$) from responses by control deer.

of 91 (74%) tests were interpreted as positive; 1 (5%) *M. bovis*–inoculated deer was interpreted as negative at every time point sampled, whereas 19 of 20 (95%) *M. bovis*–inoculated deer had at least 1 positive test at some point during the study. Twelve of 20 (60%) *M. bovis*–inoculated deer had positive test results at all time points sampled throughout the study.

Discussion

The commercial IFN-γ assay known as Cervigam™ was developed using monoclonal antibodies to IFN-γ from red deer. This study demonstrates that the Cervigam™ assay also detects IFN-γ of white-tailed deer. This is consistent with findings in the authors' laboratory and with those of others that show Cervigam™ can be used to measure IFN-γ from reindeer (*Rangifer tarandus*; data not shown), elk (*C. elaphus*; data not shown), and sambar deer (*C. unicolor*).[12] Pokeweed mitogen was found to be the best antigen of those tested for stimulation of IFN-γ production in white-tailed deer and therefore a logical choice for a positive control. Similar testing has shown that PWM also functions well as a positive control when blood from elk or reindeer is tested (data not shown). However, species differences likely exist and other deer species should be tested to determine the optimal antigen to use in positive-control samples.

This study also shows that the release of IFN-γ in response to PPDb correlates well with infection status and that this assay may be a useful means of diagnosis of *M. bovis* infection in white-tailed deer. In contrast to tuberculin skin testing, such a blood-based assay allows testing without handling the animals twice and allows repeated testing if necessary without compromising results. In cattle, tuberculin skin testing can result in desensitization of the animal for at least 60 days, thus precluding repeat testing at intervals of less than 60 days.[9]

In this study, a IFN-γ response to *M. avium* antigens was seen in *M. bovis*–inoculated deer. Cross-reactive antibody responses to *M. bovis* and *M. avium* antigens have also been seen in *M. bovis*–infected deer.[14] Similar cross-reacting responses have been demonstrated

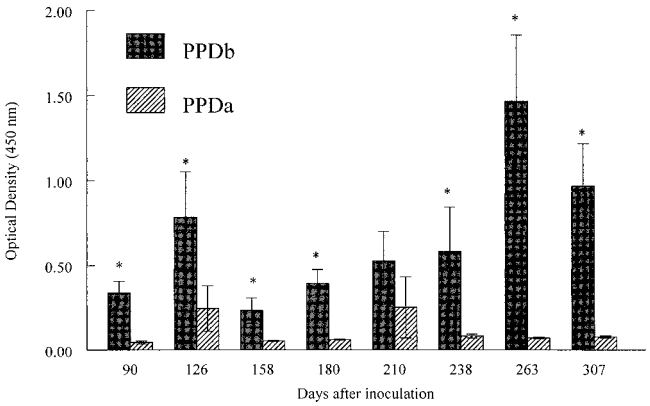

Figure 5. Interferon-γ production in response to stimulation with PPDb or PPDa as measured by OD (450 nm) using a commercial assay[c] for cervid IFN-γ. Deer were infected by intratonsillar instillation of 300 CFU of *M. bovis.* Background OD values (stimulation with PBS alone) have been subtracted from the response to PPDb or PPDa. Deer, $n = 20$ on day 90, $n = 15$ on days 126, 158, and 180, $n = 10$ on days 210, 238, and 263, $n = 5$ on day 307. *Responses to PPDb differ ($P < 0.05$) from responses to PPDa.

in *M. bovis*–infected cattle; however, as in this study, the response to homologous *M. bovis* antigen was always substantially greater.[17] In this study, differential responses allowed the identification of *M. bovis*–infected deer. Further studies, however, will be needed to characterize the IFN-γ response in deer infected with *M. avium* or other nontuberculous mycobacteria or deer coinfected with these nontuberculous mycobacteria and *M. bovis.*

Early recommendations from the manufacturer of the kit suggest that antigenic stimulation of blood samples be done within 12 hours of collection. This requirement could limit the usefulness of the test in the field. However, 1 study using the IFN-γ assay for cattle,[h] produced by the same manufacturer, has shown that a 24-hour delay in stimulation of the blood with antigen did not alter the diagnostic interpretation of the test.[16] In the present study, the effect of a similar delay in processing was not evaluated.

It is possible that the measurement of IFN-γ production as a means of antemortem diagnosis of *M. bovis* infection in deer could be used as a single test or in conjunction with intradermal skin testing. In a field evaluation of naturally infected cattle, the IFN-γ assay had a sensitivity of 81.8% and a specificity of 99.1% compared with a sensitivity and specificity of 61.8% and 96.7%, respectively, for intradermal skin testing. Interestingly, when skin testing was combined with the IFN-γ assay, the sensitivity and specificity were found to be 90.9% and 95.8%, respectively.[19] Similar studies in deer to directly compare the IFN-γ assay with intradermal skin testing, separately or combined, have not been conducted.

In studies using cattle, an increase in IFN-γ production has been noted in sensitized animals in the period 3–28 days after skin testing with PPDb.[16] It is likely that a similar rise would be seen in *M. bovis*–infected deer. However, in the present study, samples were not collected at time points that would accurately identify such an increase or characterize the duration of such an effect from skin testing. Further studies will be needed to characterize the effect of skin testing with PPDb on IFN-γ production by *M. bovis*–infected deer.

Sources and manufacturers

a. CSL Limited, Parkville, Victoria, Australia.
b. Sigma Chemical Co., St. Louis, MO.
c. Cervigam,™ Biocor Animal Health, Omaha, NE.
d. Mobay Corporation, Shawnee, KS.
e. Fort Dodge Laboratories, Fort Dodge, IA.
f. Lloyd Laboratories, Shenandoah, IA.
g. National Veterinary Services Laboratories, USDA, Ames, IA.
h. Bovigam,™ Biocor Animal Health, Omaha, NE.

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