Rapid detection of serum antibody by DPP VetTB assay in white-tailed deer infected with *Mycobacterium bovis*


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**Running title:** DPP VetTB antibody assay for deer
ABSTRACT

Bovine tuberculosis (TB) in cervids remains a significant problem affecting farmed herds and wild populations. Traditional skin testing has serious limitations in certain species, whereas emerging serological assays showed promising diagnostic performance. Recently developed immunochromatographic DPP VetTB assay has two antigen bands, T1 (MPB83 protein) and T2 (CFP10/ESAT-6 fusion protein), for antibody detection. We evaluated diagnostic accuracy of this test using serum samples collected from groups of white-tailed deer experimentally inoculated with Mycobacterium bovis, M. avium subsp. paratuberculosis, or M. bovis BCG Pasteur. In addition, we used serum samples of farmed white-tailed deer from herds with no history of TB as well as from free-ranging white-tailed deer culled during field surveillance studies performed in Michigan known to have bovine TB in the wild deer population. DPP VetTB assay detected antibody responses in 58.1% of experimentally infected animals within 8-16 weeks post-inoculation and in 71.9% of naturally infected deer, resulting in the estimated test sensitivity of 65.1% and specificity of 97.8%. The higher seroreactivity found in deer with naturally acquired M. bovis infection was associated with increased frequency of antibody responses to ESAT-6 and CFP10 proteins, resulting in greater contribution of these antigens, in addition to MPB83, to detecting seropositive animals, as compared with experimental M. bovis infection. Deer experimentally inoculated with either M. avium subsp. paratuberculosis or M. bovis BCG Pasteur did not produce cross-reactive antibodies that could be detected by the DPP VetTB assay. Present findings demonstrate the relatively high diagnostic accuracy of the DPP VetTB test for white-tailed deer, especially in detecting naturally infected animals.
INTRODUCTION

*Mycobacterium bovis*, the cause of tuberculosis (TB) in cattle, can also infect a broad range of other mammalian host species including cervids. Free-ranging deer are known to play a role as wildlife reservoirs of *M. bovis* infection (1, 2), whereas farmed deer are reportedly involved in disease transmission to cattle (3, 4) and to humans (5, 6). In the last decade, *M. bovis* outbreaks in captive cervids have been increasingly found in the United States (US), including multiple herds of white-tailed deer (*Odocoileus virginianus*) in Michigan, elk (*Cervus canadensis*) in Indiana, a mixed herd of red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) in New York, and a farm of elk and fallow deer in Nebraska (7-9).

In captive cervids, bovine TB control relies primarily on intradermal tuberculin testing and more rarely on slaughter surveillance. Skin test procedures, however, have not been fully validated for use in various cervid species. These limitations were clearly demonstrated in the recent *M. bovis* outbreak in farmed elk and fallow deer in Nebraska, where only 3/28 animals which had gross lesions and produced positive culture results were reactors in the single cervical skin test (9).

Recent studies have shown the potential of emerging antibody assays for TB detection in various cervid species (10-12). DPP VetTB assay was developed using Chembio dual path platform (DPP) technology for rapid detection of specific antibody in the laboratory or, if needed, animal-side under field conditions. This immunoassay has been recently evaluated in elk, red deer, and fallow deer (9, 11, 13). In the present report, we describe the diagnostic performance of DPP VetTB assay in white-tailed deer experimentally or naturally infected with *M. bovis*.
MATERIALS AND METHODS

Animals. White-tailed deer (1-3 years of age) were raised within a TB-free herd at the National Animal Disease Center (NADC), Ames, Iowa, USA, or obtained from farms with no history of TB. Study groups included 24 non-inoculated negative control animals, 5 deer vaccinated subcutaneously with *M. bovis* BCG Pasteur (~5 x 10^7 colony-forming units (CFU), two doses, 6-week interval between injections), 3 animals inoculated orally with *M. avium* subsp. *paratuberculosis* strain K10 (MAP, ~2 x 10^7 CFU), and 31 deer infected by aerosol or intratonsilarly with various doses of *M. bovis* (3x10^2 to 2x10^8 CFU), as described previously (14, 15). Serum samples were collected serially at various time points after vaccination / challenge and stored frozen at -70°C until use in serological tests. Animals were euthanized 3 to 6 months after *M. bovis* inoculation or BCG vaccination or up to 18 months after MAP inoculation. Various tissues were collected for bacteriologic culture and microscopic examination. Disease was confirmed at necropsy for each *M. bovis*- and *MAP*-infected deer by the presence of gross lesions, histopathology, and mycobacterial culture. The Institutional Animal Care and Use Committee approved protocols detailing procedures and animal care prior to initiation of the experiments.

In addition, serum samples were collected from 483 free-ranging white-tailed deer in Michigan, a region in the US where *M. bovis* infection in this host species is endemic (16). The animals enrolled in the study inhabited the “core” of the bovine TB outbreak area (1). Blood specimens of variable quality were obtained during 2004-2010 from (a) hunter-harvested deer, (b) carcasses presented to wildlife disease laboratory during
routine surveillance, and (c) depopulation of a fenced deer shooting preserve, as
described previously (17). All animals were examined for gross lesions consistent with
TB using the standardized protocols (17), followed by histopathology and mycobacterial
culture from various tissue specimens including lungs, parietal pleura, and medial
retropharyngeal lymph nodes.

**Dual path platform (DPP) VetTB assay.** The DPP format is a two-step test
designed for rapid antibody detection in multiple host species (18-20) including cervids
(9, 11, 13). The assay has two test antigen bands on the membrane strip, T1 (MPB83
protein) and T2 (CFP10/ESAT-6 fusion), for differential IgG antibody detection by
colloidal gold particles coupled with hybrid protein A/G. The DPP VetTB assay was
performed as previously described (18) using 5 μl of serum, 2 drops of buffer added to the
sample well, and 4 drops of buffer added to the conjugate well. Results were evaluated at
15 minutes visually, with a band of any intensity being read as an antibody positive
reaction. No visible reactivity with any of the two test antigens was interpreted as an
antibody negative result. In addition, a DPP optical reader device was used to measure
reflectance in relative light units (RLU). Based on extensive analyses performed with
sera from known TB-free deer, reactivity of T1 and/or T2 above cut-off value of 50 RLU
was considered an antibody positive result.

**Multiantigen print immunoassay (MAPIA).** The testing was performed as
previously described (14). The antigen panel consisted of 12 recombinant proteins of *M.
tuberculosis* and 2 native antigen preparations of *M. bovis* as follows: ESAT-6 and
CFP10 proteins as well as hybrids CFP10/ESAT-6 and Acr1/MPB83 from Statens Serum
Institut (Copenhagen, Denmark); MPB59, MPB64, MPB70, and MPB83 proteins as well
as bovine protein purified derivative (B-PPD) tuberculin and *M. bovis* culture filtrate (MBCF) from the Veterinary Sciences Division (Stormont, UK); Mtb8 and polyepitope fusion TBF10 developed by Corixa Corp. (Seattle, WA, USA); alpha-crystallin (Acr1) and the 38 kDa protein from Standard Diagnostics (Seoul, South Korea). Deer IgG antibody bound to the immobilized antigens was detected by peroxidase-conjugated protein G (Sigma, St. Louis, MO, USA) diluted at 1:1000 and visualized with 3,3’,5,5’-tetramethyl benzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). MAPIA results were evaluated visually, with a band of any intensity being read as an antibody positive reaction.

**Data analysis.** Diagnostic performance of the DPP VetTB assay was evaluated against the gold standard of *M. bovis* culture by calculating test sensitivity and specificity using available software (21) and presented with the 95% confidence intervals (CI). For specificity estimates in the NADC studies, we used sera from the non-infected control group and pre-inoculation samples from the experimentally infected or vaccinated animals. MAP-inoculated and BCG-vaccinated deer were used for serological cross-reactivity testing and not included in the specificity evaluation.

**RESULTS AND DISCUSSION**

**Antigen recognition by antibodies during *M. bovis* infection.** Use of multiple antigens enabled detailed analysis of antibody profiles observed in infected white-tailed deer. Figure 1 shows typical antigen reactivity patterns obtained with representative sera from deer experimentally inoculated with *M. bovis*. In MAPIA, MPB83 protein was the most frequently recognized antigen by serum antibodies. The Arc1/MPB83 chimical
molecule and MBCF (both containing MPB83 epitopes) were also reactive with the
MPB83-antibody positive sera. ESAT-6 and CFP10 proteins, as well as the
CFP10/ESAT-6 fusion protein, were reactive as well, albeit, in fewer infected deer. No
bands were detected with pre-inoculation sera (Figure 1, strips 1a and 2a). The antibody
profiles were generally variable from animal to animal, irrespective of the route of M.
bovis inoculation (aerosol vs. intratonsilar). The MAPIA findings for MPB83 and
CFP10/ESAT-6 proteins were consistent with the DPP VetTB data obtained for T1 and
T2, respectively (Figure 1); thus, demonstrating agreement between the two
immunoassays.

Antibody responses detected in the Michigan wild deer with naturally acquired M.
bovis infection appeared stronger and involved more reactive bands in MAPIA (Figure
2), compared to the experimentally inoculated animals (Figure 1). As with experimentally
infected deer, the MPB83 protein was recognized by most of culture-confirmed free-
ranging deer. However, the reactivity of CFP10 and ESAT-6 antigens was much greater
than that found during the experimental M. bovis infection. With generally heterogeneous
antibody profiles observed in deer with naturally acquired disease, serological responses
obtained from sera collected during different winter seasons did not reveal year-to-year
differences. As with the experimental M. bovis infection, DPP VetTB assay results
correlated well with the MAPIA data, confirming the higher contribution of T2
(CFP10/ESAT-6) to the antibody detection in the Michigan wild deer (Figure 2) versus
experimentally-infected deer (Figure 1).

Based on cattle studies, it is generally believed that immune responses to
experimental M. bovis infection may be stronger than those usually found in naturally-
acquired bovine TB (22). Therefore, diagnostic sensitivity of novel immunoassays may be overestimated if evaluated only on experimentally generated specimens, requiring further validation using field samples. In the present study, however, we found that Michigan deer with natural *M. bovis* infection produced more robust antibody responses, especially to ESAT-6 and CFP10 proteins (T2 in DPP VetTB assay), than did experimentally-inoculated deer of the same species. This unexpected finding may be explained by the short duration design of the *M. bovis* inoculation experiments, not conducive for full maturation of the antibody response to infection. It is possible that most of the Michigan wild deer that developed TB represented more advanced stages of disease or had been infected longer. Consequently, their immune responses were generally stronger and involved more antibody-reactive antigens. Further studies are necessary to confirm this assumption.

**Kinetics of antibody responses to *M. bovis* infection.** Serial specimens collected during experimental *M. bovis* infection were tested to determine seroconversion times. Most deer developed detectable responses between 8 and 16 weeks post-inoculation, followed by gradually increasing antibody levels during several weeks, and fluctuating but generally sustainable seroreactivity thereafter. Figure 3 shows an example of the individual IgG kinetics measured by DPP VetTB assay in deer #571 that was infected with *M. bovis* intratonsilarly. The serological response involved both T1 and T2 reactivity in this animal suggesting that antibody levels to each of the test antigens (MPB83 and CFP10/ESAT-6, respectively) evolved independently over the course of infection, resulting in variable quantitative seroreactivity profiles observed over time. These findings indicate that *M. bovis* infection can induce measurable IgG responses in cervids.
within 2-3 months post-inoculation, thus supporting previously reported data obtained by MAPIA in experimentally infected white-tailed deer (14), elk (23), and reindeer (24).

**Diagnostic performance of DPP VetTB assay.** Taking into account the serological differences found by MAPIA between the experimentally infected animals and the wild deer with naturally acquired *M. bovis*, the accuracy of the DPP VetTB assay was estimated for each of these groups separately (Table 1). In free-ranging deer, the test sensitivity was higher (71.9%) than in the experimentally infected animals (58.1%), whereas the specificity was nearly identical between the two groups (98.2% and 98.4) and close to that found for the farmed deer (97.2%) in the present study. When all animals (63 with TB and 903 controls) were combined, the resulting test sensitivity was 65.1% (95% CI, 51.9-76.4) and specificity was 97.8% (95% CI, 96.5-98.6).

The wild deer used in the Michigan study to determine DPP VetTB assay specificity included 5 animals with granulomatous lesions in the lungs which were all negative for *M. bovis* by culture and PCR. None of these deer showed antibody positive results in the DPP VetTB assay. The specificity was further evaluated by testing serum samples from 3 deer experimentally inoculated with *MAP* and 5 deer vaccinated with *M. bovis* BCG Pasteur. No antibody cross-reactivity was found by DPP VetTB assay in these animals, despite development of strong serological responses by the *MAP*-inoculated deer that were detected by ELISA using lipoarabinomannan-enriched antigen (15). It is known that, in contrast to virulent *M. bovis*, BCG lacks ESAT-6 and CFP10 antigens (25). Further, expression of MPB70 and MPB83 proteins varies significantly amongst the BCG strains, with BCG strain Pasteur (the vaccine used in the current study) being a low-MPB83/70-producer (26). High specificity of the DPP VetTB assay was also shown for...
other cervid species (9, 11, 13). In these reports, the test sensitivity was 79% for captive elk involved in the *M. bovis* outbreak in the US (Nebraska) and 85% for red deer in New Zealand, whereas for fallow deer sensitivity estimates varied from 71% in Spain (wild population) to 91% in the US (captive herd in Nebraska). Thus, the test sensitivity determined in the present study for white-tailed deer with naturally acquired *M. bovis* infection (72%) appears within the range of sensitivity estimates reported for other cervid species.

Comparison of the antibody detection with the tuberculin test for detection of bovine TB in deer was not among the objectives of this report. Free-ranging deer were not skin tested in Michigan, whereas experimentally infected animals were all tuberculin reactors and, therefore, could not represent natural infection in the cervid population. Previous studies, however, have demonstrated the added value of serology in situations where the intradermal tuberculin test failed to detect most of the *M. bovis*-infected deer in the affected herd (9). Antibody assays may also be used to supplement skin test procedures for the detection of *M. bovis*-infected animals (12, 13).

In conclusion, the serologic approach to identify *M. bovis*-infected cervids is increasingly gaining recognition for use in bovine TB control programs. Published data have demonstrated that antibody detection assays may provide useful ancillary tools for improved bovine TB control in both cattle and cervids. The present study showed relatively high diagnostic accuracy of the DPP VetTB test for white-tailed deer, especially in detecting naturally infected animals. Further evaluation studies are needed to validate this test utility in other cervid species.
ACKNOWLEDGMENTS

The authors are grateful to Claudia Quinn and Laurie Nussbaum for skillful technical assistance with the serological assays as well as Rebecca Lyon, Jessica Pollock, Shelly Zimmerman, Mike Howard, Kristin Bass, and the animal care and clinical veterinary staff at NADC.
REFERENCES


Table 1. Diagnostic performance of DPP VetTB assay in white-tailed deer

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NADC, National Animal Disease Center; CI, confidence interval.
FIGURE LEGENDS

Figure 1. Antibody responses detected by MAPIA and DPP VetTB assay in white-tailed deer experimentally infected with *M. bovis*. Results are shown for sera collected from 8 representative animals before (1a, 2a) and 10 to 20 weeks (1b, 2b, 3-8) after *M. bovis* inoculation. Strip images show MAPIA results. Pre-printed antigens are indicated in the right margin. Semi-quantitative DPP VetTB assay data obtained with the same sera for T1 (MPB83 reactivity) and T2 (CFP10/ESAT-6 reactivity) by a reflectance reader are presented under the strips. Reading values above 50 RLU (shown in bold) were considered as antibody positive results. Arrows show positions of DPP VetTB T1 and T2 antigens on MAPIA strips.

Figure 2. Antibody responses detected by MAPIA and DPP VetTB assay in free-ranging white-tailed deer naturally infected with *M. bovis*. Results are shown for 12 representative culture-positive animals culled in Michigan in three different winter seasons (years indicated in top margin). Strip images show MAPIA results. Pre-printed antigens are indicated in the right margin. Semi-quantitative DPP VetTB assay data obtained with the same sera for T1 (MPB83 reactivity) and T2 (CFP10/ESAT-6 reactivity) by a reflectance reader are presented under the strips in the lower margin. Reading values above 50 RLU (shown in bold) were considered as antibody positive results for the DPP VetTB assay. Arrows show positions of DPP VetTB T1 and T2 antigens on MAPIA strips.
Figure 3. Kinetics of the antibody response to T1 and T2 antigens used in DPP VetTB assay by deer #571 intratonsilarly inoculated with *M. bovis*. Graph shows kinetics of the antibody levels to MPB83 protein (T1, circles) and CFP10/ESAT-6 fusion antigen (T2, triangles) measured in RLU by DPP reader in serial serum samples collected during experimental infection. Time post-inoculation is shown in weeks in the lower margin.
Figure 1.

M. bovis inoculation

aerosol

intratrnsilar

T1 →

T2 →

T1: 6 66 0 632 583 388 193 629 23

T2: 0 370 0 0 635 0 0 1 23

-- ESAT6
-- CFP10
-- Mtb8
-- MPB64
-- MPB59
-- MPB70
-- MPB83
-- Acr-1
-- 38kDa
-- CFP10/ESAT6
-- TFB10
-- Acr1/MPB83
-- B-PPD
-- MBCF

DPP
VetTB
Figure 2.