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. The recently developed immunochromatographic dual-path platform (DPP) VetTB assay has two antigen bands, T1 (MPB83 protein) and T2 (CFP10/ESAT-6 fusion protein), for antibody detection. We evaluated the diagnostic accuracy of this test by 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 from farmed white-tailed deer in herds without a 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. The DPP VetTB assay detected antibody responses in 58.1% of experimentally infected animals within 8 to 16 weeks postinoculation and in 71.9% of naturally infected deer, resulting in an estimated test sensitivity of 65.1% and a specificity of 97.8%. The higher seroreactivity found in deer with naturally acquired M. bovis infection was associated with an increased frequency of antibody responses to the ESAT-6 and CFP10 proteins, resulting in a greater contribution of these antigens, in addition to MPB83, to the detection of seropositive animals, 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. The present findings demonstrate the relatively high diagnostic accuracy of the DPP VetTB test for white-tailed deer, especially in the detection of naturally infected animals.

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, 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 that 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). The dual-path platform (DPP) VetTB assay was developed by using Chembio DPP technology for the rapid detection of a 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 the DPP VetTB assay in white-tailed deer experimentally or naturally infected with M. bovis.

**Materials and Methods**

**Animals.** White-tailed deer (1 to 3 years of age) were raised within a TB-free herd at the National Animal Disease Center (NADC), Ames, IA, or obtained from farms with no history of TB. Study groups included 24 noninoculated negative-control animals, 5 deer vaccinated subcutaneously with M. bovis BCG Pasteur (~5 × 10^7 CFU, two doses, 6-week interval between injections), 3 animals inoculated orally with M. avium subsp. paratuberculosis strain K10 (~2 × 10^7 CFU), and 31 deer infected by aerosol or intratonsilarly with various doses of M. bovis (3 × 10^5 to 2 × 10^6 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 M. avium subsp. paratuberculosis strain K10 inoculation. Various tissues were collected for bacteriologic culture and microscopic examination. Disease was confirmed at necropsy for each M. bovis- and M. avium subsp. paratuberculosis strain K10-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 the initiation of the experiments.

In addition, serum samples were collected from 483 free-ranging white-tailed deer in Michigan, a region of the United States where M. bovis...

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infection in this host species is endemic (16). The animals enrolled in this study inhabited the “core” of the bovine TB outbreak area (1). Blood specimens of variable quality were obtained during 2004 to 2010 from (i) hunter-harvested deer, (ii) carcasses presented to a wildlife disease laboratory during routine surveillance, and (iii) depopulation of a fenced deer shooting preserve, as described previously (17). All animals were examined for gross lesions consistent with TB in accordance with the standardized protocols (17), followed by histopathology and mycobacterial culture from various tissue specimens, including lungs, parietal pleura, and mediastinal lymph nodes.

**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), with 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 visually at 15 min, 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).

On the basis of extensive analyses performed with sera from deer known to be TB free, reactivity of T1 and/or T2 above a cutoff value of 50 RLU was considered an antibody-positive result.

**Multiantigen print immunoassay (MAPIA).** Testing was performed as previously described (14). The antigen panel consisted of 12 recombinant proteins of *M. tuberculosis* and two native antigen preparations of *M. bovis* as follows: the ESAT-6 and CFP10 proteins, as well as hybrids CFP10/ESAT-6 and Acr1/MPB83, from the Statens Serum Institut (Copenhagen, Denmark); the MPB59, MPB64, MPB70, and MPB83 proteins, as well as bovine protein purified derivative tuberculin and *M. bovis* culture filtrate (MBCF), from the Veterinary Sciences Division (Stormont, United Kingdom); Mtb8 and polypeptide fusion TBE10 developed by Corixa Corp. (Seattle, WA); and 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) diluted 1:1,000 and visualized with 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD). MAPIA results were evaluated visually, with a band of any intensity being read as an antibody-positive reaction.

**Data analysis.** The diagnostic performance of the DPP VetTB assay was evaluated against the gold standard of *M. bovis* culture by calculating test sensitivity and specificity with available software (21) and is presented with the 95% confidence intervals (CIs). For specificity estimates in the NADC studies, we used sera from the noninfected control group and preinoculation samples from the experimentally infected or vaccinated animals. *M. avium* subsp. *paratuberculosis* strain K10-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.

The use of multiple antigens enabled a detailed analysis of the 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 the MAPIA, the MPB83 protein was the antigen most frequently recognized by serum antibodies. The Arc1/MPB83 chimeric molecule and MBCF (both containing MPB83 epitopes) were also reactive with the MPB83 antibody-positive sera. The 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 preinoculation sera (Fig. 1, strips 1a and 2a). The antibody profiles were generally variable from animal to animal, irrespective of the route of *M. bovis* inoculation (aerosol versus intratonsilar). The MAPIA findings for the MPB83 and CFP10/ESAT-6 proteins were consistent with the DPP VetTB data obtained for T1 and T2, respectively (Fig. 1), thus demonstrating agreement between the two immunoassays.

The antibody responses detected in the Michigan wild deer with naturally acquired *M. bovis* infections appeared stronger and involved more reactive bands in the MAPIA (Fig. 2) than did those of experimentally inoculated animals (Fig. 1). As with experimentally infected deer, the MPB83 protein was recognized by most of the culture-confirmed free-ranging deer. However, the reactivity of the CFP10 and ESAT-6 antigens was much greater than that found during experimental *M. bovis* infection. With generally heterogeneous antibody profiles observed in deer with naturally acquired disease, the 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 greater contribution of T2 (CFP10/ESAT-6) to antibody detection in Michigan wild deer (Fig. 2) than in experimentally infected deer (Fig. 1).

On the basis of 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, the diagnostic sensitivity of novel immunoassays may be overestimated if they are evaluated only on experimentally generated specimens, requiring further validation with field samples. In the present study, however, we found that Michigan deer with natural *M. bovis* infections produced more robust antibody responses, especially to the ESAT-6 and CFP10 proteins (T2 in the...
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, which is not conducive to full maturation of the antibody response to infection. It is possible that most of the Michigan white-tailed 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 postinoculation, followed by gradually increasing antibody levels for several weeks and fluctuating but generally sustainable seroreactivity thereafter. Figure 3 shows an example of the individual IgG kinetics measured by the DPP VetTB assay in deer 571, which was infected with M. bovis intratonsilially. The serological response involved both T1 and T2 reactivity in this animal, suggesting that levels of antibody to each of the test antigens (MPB83 and CFP10/ESAT-6 reactivity) evolved independently over the course of infection, resulting in the variable quantitative seroreactivity profiles observed over time. These findings indicate that M. bovis infection can induce measurable IgG responses in cervids within 2 to 3 months postinoculation, thus supporting previously reported data obtained by MAPIA from 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 the same in the two groups (98.2 and 98.4%) and close to that found for farmed deer (97.2%) in the present study. When all of the animals (63 with TB and 903 controls) were combined, the resulting test sensitivity was 65.1% (95% CI, 51.9 to 76.4%) and the specificity was 97.8% (95% CI, 96.5 to 98.8%).

The wild deer used in the Michigan study to determine DPP VetTB assay specificity included five 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 three deer experimentally inoculated with M. avium subsp. paratuberculosis strain K10 and five deer vaccinated with M. bovis BCG Pasteur. No antibody cross-reactivity was found by DPP VetTB assay in these animals, despite the development of strong serological responses by the M. avium subsp. paratuberculosis strain K10-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 the MPB70 and MPB83 proteins varies significantly among the BCG strains, with BCG strain Pasteur (the vaccine used in the present 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 United States (Nebraska) and 85% for red deer in New Zealand, whereas for fallow deer the sensitivity estimates varied from 71% in Spain (wild population) to 91% in the United States (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 to be 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 the identification of *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 a 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.

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