

RESEARCH ARTICLE

Open Access

Use of ethanol extract of *Mycobacterium bovis* for detection of specific antibodies in sera of farmed red deer (*Cervus elaphus*) with bovine tuberculosis

Ashutosh Wadhwa¹, Rachel E Johnson¹, Colin G Mackintosh², J Frank T Griffin³, W Ray Waters⁴, John P Bannantine⁴ and Shigetoshi Eda^{1*}

Abstract

Background: Bovine tuberculosis (bTB) in wildlife species poses a threat to domestic livestock in many situations. Control programs for bTB in livestock depend on testing and slaughtering the positive animals; however, the currently available diagnostic tests often have poor specificity. In our previous study, we developed a specific and sensitive enzyme linked immunosorbent assay (ELISA) for another mycobacterial disease – Johne’s disease, using surface antigens of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) extracted by briefly agitating the bacilli in 80% ethanol solution. The ELISA test was named ethanol vortex ELISA (EVELISA). The objective of this study is to examine whether EVELISA technique could be used to specifically detect anti-*Mycobacterium bovis* (*M. bovis*) antibodies in the serum of *M. bovis*-infected farmed red deer (*Cervus elaphus*). We tested a total of 45 red deer serum samples, divided in 3 groups – uninfected animals (n = 15), experimentally infected with *M. bovis* (n = 15) and experimentally infected with MAP (n = 15).

Results: The presence of anti-*M. bovis* antibodies was tested using an ethanol extract of *M. bovis*. Without absorption of anti-MAP cross reactive antibodies, it was found that 13 out of the 15 MAP-infected animals showed high antibody binding. Using heat killed MAP as an absorbent of cross reactive antibodies, anti-*M. bovis* antibodies were detected in 86.7% of *M. bovis*-infected animals with minor false positive results caused by MAP infection.

Conclusions: The results from this study suggest that EVELISA may form a basis for a sensitive and specific test for the diagnosis of bTB in farmed red deer.

Keywords: Bovine tuberculosis, ELISA, *Mycobacterium bovis*, Red deer

Background

Farming of red deer (*Cervus elaphus*) has been an emerging alternative livestock industry mainly in New Zealand, USA, China, Russia and Canada [1]. Being in continuous contact with the livestock and the free-ranging wildlife, farmed red deer populations are at increased risk to get and spread infectious diseases. Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (*M. bovis*), is a chronic infectious disease of international zoonotic and economic importance [2]. It is characterized by the formation of granulomatous lesions with varying degrees of necrosis,

calcification and encapsulation [3-7]. bTB has been identified in a wide range of wildlife species, domestic animals and humans [8,9]. Global economic loss due to bTB is estimated to be about US\$ 3 billion annually [10]. Since there are no effective treatments or vaccines for bTB, rigorous testing and removal of diseased animals remains the only control measure.

In contrast to the control programs for bTB in wildlife species, bTB in farmed deer is primarily monitored by skin testing and rarely by slaughter surveillance. One of the major ante mortem tests for bTB is the tuberculin skin test (TST) using purified protein derivatives (PPD) of *M. bovis* [4,11]. In the US, there is requirement of a negative skin test for interstate transport and it includes a voluntary herd accreditation program [12]. However,

* Correspondence: seda@utk.edu

¹Department of Forestry, Wildlife and Fisheries, Center for Wildlife Health, University of Tennessee Institute of Agriculture, Knoxville, Tennessee 37996, USA

Full list of author information is available at the end of the article

the participation in such programs is very low due to inadequate handling facilities and need to recapture animals for testing 72 hours after the injection of PPD [13,14]. Further, a recent study showed that interpretation of TST could be confounded by infection of red deer with environmental mycobacteria [15]. An interferon- γ release assay has also been evaluated for bTB diagnosis in capture cervids, [16,17] but the test requires fresh blood samples and also has not been validated for diagnosis of bTB in free-ranging wildlife species [18]. Antibody-based assays for detection of bTB have shown promising results due to their flexibility and cost effectiveness. Prior studies on the development of antibody based assays have used cross-reactive preparations of *M. bovis*, such as a crude cell sonicate [19] culture filtrate [20] PPD [21] and lipoarabinomannan (LAM) [22]. Specific molecules like ESAT-6, CFP10, MPB83 and MPB70 have also been used for detection of anti-*M. bovis* antibodies [23-25]. Recent studies have demonstrated the advantages of using multiple antigens (e.g. ESAT-6, CFP10 and MPB83) in multi-antigen print immune-assay (MAPIA) [12,26], lateral flow rapid test (RT) [18] or dual path platform (DPP) [27] assays. Although these studies have shown promising results in detecting antibodies against *M. bovis*, the presence of anti-*Mycobacterium avium* ssp. *paratuberculosis* (MAP) antibodies due to confounding factors like infection and/or vaccination may cause interference in interpretation [28].

We have previously developed a novel enzyme-linked immunosorbent assay (ELISA), called an ethanol vortex ELISA (EVELISA) using surface antigens of MAP for detecting anti-MAP antibodies in serum at early stages of Johne's disease (JD) [29-32]. The aim of the present work was to assess the performance of EVELISA optimized to diagnose bTB using serum samples from various groups of red deer (*Cervus elaphus*) including animals experimentally infected with *M. bovis* or MAP.

Methods

Samples

In order to evaluate the performance of EVELISA, a total of 45 red deer sera were obtained from 3 different studies in New Zealand. The first group (Uninfected) consisted of 15 deer approximately 12 months old, which were not challenged with either of *M. bovis* or MAP. All the animals in this group were culture negative for *M. bovis* using lymph node samples and blood samples taken one week prior to slaughter were all serologically negative for MAP using an IgG₁ ELISA test (Paralisa™, Disease Research Laboratory (DRL), Department of Immunology and Microbiology, University of Otago, Dunedin, NZ) [33]. The second group (*M. bovis* infected) consisted of 15 deer approximately 12 months old, which had been experimentally challenged using 0.2 mL volume of 500 CFU of *M. bovis*

into the left tonsillar crypt of anesthetized deer [34]. *M. bovis* was isolated at slaughter from gross lesions or pooled lymph node samples (head, thoracic or intestinal lymph nodes) from all 15 deer 27 weeks after experimental challenge. All blood samples were tested using an ELISA Tb test called EBT [35] and a comparative cervical tuberculin test (CCT) [36]. For the CCT, intradermal injections of 0.1 mL of avian tuberculin (2500 IU; A) and bovine tuberculin (5000 IU; B) were given at two closely clipped sites on the neck. Skin thickness was measured before injection and 72 hours later. The CCT is considered positive if the increase at site B is greater than or equal to site A; and, negative if site A is greater than site B. All the animals in the second group (*M. bovis* infected) were tested positive by CCT. Serum samples of 11 out of the 15 animals in this group were positive by ETB. Of the 15 blood samples collected a week prior to slaughter, 7 samples were seropositive for MAP using the Paralisa™ test. Finally, the third group (MAP infected) consisted of 15 deer experimentally infected with MAP as previously described [33]. All the samples in this group were from animals sourced from a property with no history of bTB or JD. MAP was isolated from all the deer in this group by culture after 50 weeks post infection and 12 out of the 15 samples collected immediately before slaughter were seropositive using the Paralisa™ test. Serum samples of 10 out of the 15 animals in this group were positive by ETB, showing high false positive rate in MAP infected animals. Animal use described in this study was approved by the AgResearch Invermay Ethics Committee (AEC11115).

EVELISA

A virulent strain of *M. bovis* (HC2005T), which was originally isolated from an *M. bovis* infected dairy cow, was cultured in Middlebrook's 7H9 medium (Becton Dickinson, Cockeysville, MD) with addition of 0.05% Tween 80 (Fisher Scientific, Fair Lawn, NJ), 10% oleic acid-albumin-dextrose-NaCl (Becton Dickinson, Microbiology Systems, Franklin Lakes, NJ) at 37°C. For antigen preparation *M. bovis* bacilli were harvested from stationary phase cultures, and centrifuged at 2,600 \times g for 10 minutes; the pellet was then suspended in 80% ethanol and agitated by vortex at room temperature for 2 min, and centrifuged at 10,621 \times g for 10 minutes to dislodge surface antigens. Extracted *M. bovis* antigen was diluted in the ethanol solution and 50 μ L of the solution was immobilized on wells of a 96-well plate by evaporation. MAP (K10 strain) was used for preabsorption step in this study. The antigen-coated plate was incubated with 150 μ L of buffer B (10 mM phosphate buffered saline, pH 7.0, containing 0.05 v/v% Tween 20 and 10 v/v% SuperBlock [PIERCE Biotechnology, Rockford, IL]) at

room temperature for 30 min. The plate was then washed 4 times with 200 μ L of PBST (10 mM phosphate buffered saline, pH 7.0, containing 0.05% Tween 20). Fifty μ L of serum sample (with or without preabsorption of cross-reactive antibodies with heat-killed MAP [K10 strain, 4 mg/mL] for 30 minutes) was then inoculated and incubated at room temperature for one hour [32]. After washing the wells four times with 200 μ L of PBST, 50 μ L of horseradish peroxidase (HRP)-conjugated rabbit anti-deer IgG heavy and light chains (1:1000 dilution; Kirkegaard & Perry Laboratories, Inc. Gaithersburg, MD, diluted in buffer B) was added in each well and incubated at room temperature for one hour. After washing the wells four times with 200 μ L of PBST, 100 μ L of tetramethylbenzidine (TMB) solution (Thermo Scientific, Rockford, IL) was used to develop color reaction according to manufacturer's instruction and optical density (OD) of the solution was determined by a microplate reader (Model 680, BioRad, Hercules, CA) at 450 nm for 10 min after terminating the reaction by adding 100 μ L of 2 M sulfuric acid.

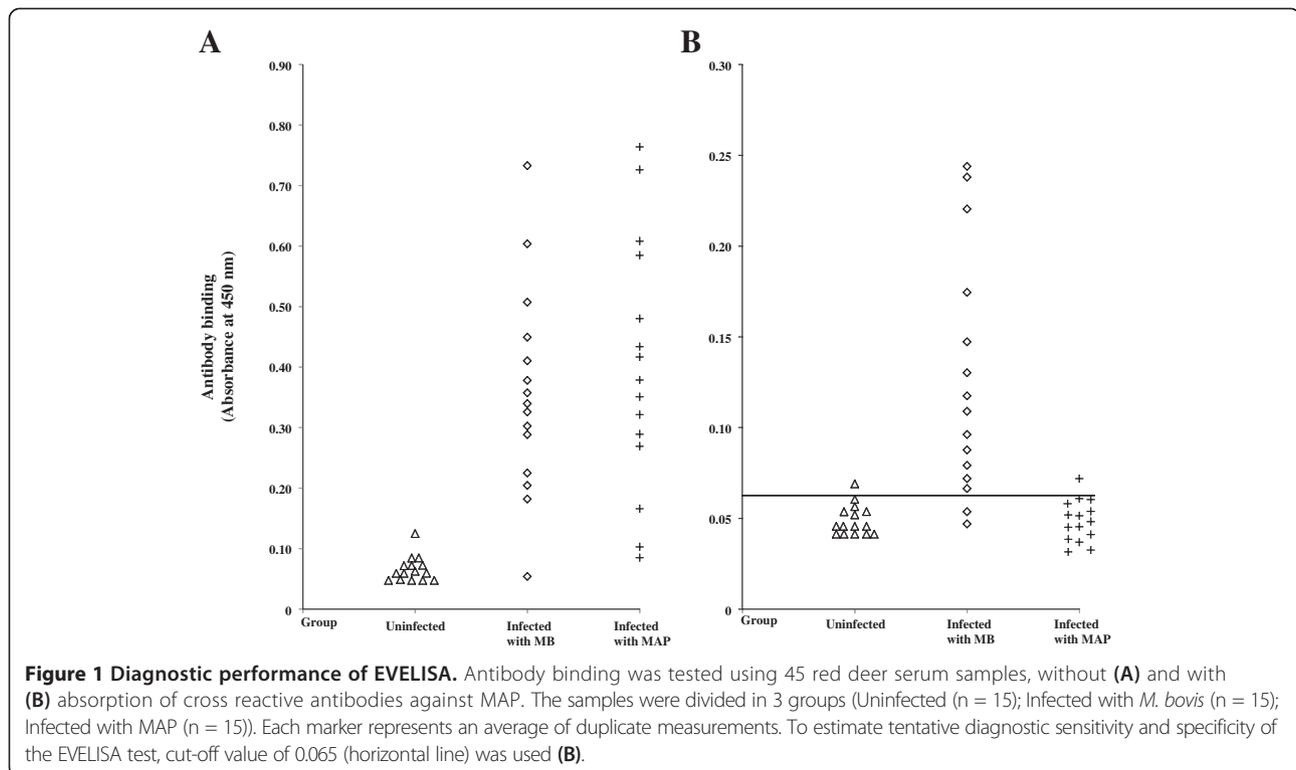
Statistical analysis

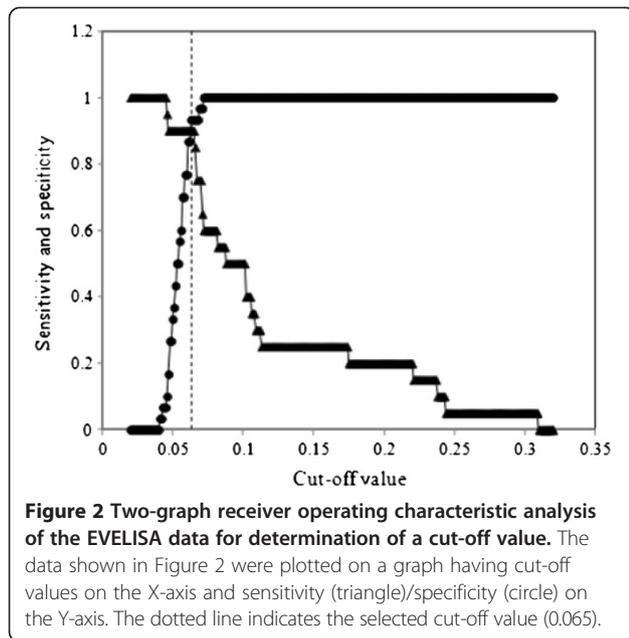
All experiments were conducted in duplicate or triplicate, and repeated at least twice. The test sensitivity was determined by dividing the number of *M. bovis* culture-test positive animals by the total number of *M. bovis* culture positive animals, with the result expressed as a percentage. The test specificity was determined by

dividing the number of bTB free, test-negative animals by the total number of bTB free animals, with the result expressed as a percentage. The cut-off value was determined based on the two-graph receiver operating characteristic analysis [37].

Results

The performance of EVELISA test to detect anti-*M. bovis* antibodies in the sera of red deer was evaluated and compared with or without the use of MAP as an absorbent for cross-reactive antibodies (Figure 1A-B). Without pre-absorption of sera with MAP (Figure 1A), 14 out of 15 *M. bovis* positive samples (*M. bovis*-infected) showed higher antibody binding levels than those in any of uninfected samples (Uninfected). In the same figure, 13 out of 15 samples in the third group (MAP-infected) showed high antibody binding levels than those in the uninfected samples, showing a high rate of false positive reactions. In Figure 1B, antibody binding was tested after absorbing cross-reactive antibodies with heat-killed MAP bacilli. Antibody binding levels in the MAP-infected groups were significantly reduced. Two-graph receiver operating characteristic analysis was used to calculate a cut-off value of 0.065 (Figure 2). Anti-*M. bovis* antibodies were detected in 86.7% (13/15) of *M. bovis*-infected animals. Out of the 15 animals in the first group (Uninfected), 14 animals showed negative reaction with the EVELISA. The most significant difference was that antibody binding levels in the third





group (n = 15, MAP infected) were reduced to the same levels as the first group (Uninfected) and only one sample in the MAP infected group showed antibody level higher than the cut-off value. We also tested 5 animals naturally infected with *M. bovis* and found that all the animals had higher antibody binding than the cut-off value (Mean \pm standard deviation of absorbance at 450 nm = 0.127 ± 0.103).

Discussion

Previous studies on developing ELISA for bTB in deer have used PPD (bovine and avian), MPB70 [4,25,38], and LAM [39,40]. Griffin *et al.* [35] have described the use of bovine and avian PPD in comparative ELISA format ($B > A$) and suggested a sensitivity of 70% [35]. They have also reported an ancillary blood test (BTB) which is a composite test of both lymphocyte transformation assay and the bovine PPD ELISA. The BTB showed a high sensitivity of 94%; however, the test is a costly assay compared to ELISA and is used as an ancillary test to examine TST-reactors rather than for whole herd testing. LAM has also been used as an antigen in development of antibody based assays and to evaluate antibody response kinetics on experimental inoculation but it is cross reactive to antibody elicited by non-tuberculous mycobacteria [39]. Waters *et al.* [40] suggested the use of purified proteins to improve the specificity of antibody based assays [40].

Recently, other serologic tests have been developed – MAPIA, RT and dual-path-platform (DPP) VetTB assay – using *M. bovis*-specific antigens such as MPB83, ESAT6 and CFP-10 antigens [18,26,41]. Sensitivities of MAPIA

and Cervid TB STAT-PAK were estimated to be 76.7% and 72.5%, respectively, by using sera of cervids (*C. elaphus*) experimentally infected with *M. bovis* [42]. Buddle *et al.* [28] compared the 2 lateral flow tests – CervidTB STAT-PAK and DPP VetTB assay and reported specificities of 83.8% and 91.4%, respectively [28]. Further, in a following study, higher sensitivities of CervidTB STAT-PAK (82%) and DPP VetTB (79%) were reported by Waters *et al.* [12] by using sera of *C. elaphus* naturally infected with *M. bovis* [12]. Boadella *et al.* [43] reported a sensitivity of 51% and specificity of 96% for fallow deer using bovine PPD ELISA. They also tested DPP VetTB assay and depending on the cut-off value selected, the sensitivity and specificity ranged from 62-71% and 88-95%, respectively [43]. Although these rapid tests show improved sensitivity and specificity, preparation of the highly purified recombinant proteins can be costly.

In this study, we used *M. bovis* antigens prepared simply by agitating the bacteria in an ethanol solution. Our previous studies showed that ethanol extract of MAP contains MAP-specific antigens and can be used to diagnose JD with high sensitivity [30,44]. We also showed that absorption of cross reactive antibodies in serum samples with *Mycobacterium phlei* improved specificity of the EVELISA test for JD [32]. This is similar to the observation in this study that specificity of EVELISA test could be improved by absorption of cross reactive antibodies by using environmental mycobacteria. After absorption of cross reactive antibodies with MAP, anti-*M. bovis* antibodies were detected in 86.7% of *M. bovis*-infected animals with only a minor false positive result in uninfected animals. Fourteen out of 15 samples from animals in the group (MAP infected) were tested negative by the EVELISA, indicating that a majority of antibodies reacting with MAP were removed by the absorption. Thus, the results of this study suggest that the EVELISA could diagnose bTB in MAP-infected red deer with minimum false positive results caused by antibodies reacting with MAP, encouraging further studies to validate the test using a larger number of samples obtained from red deer farms. In such studies, it would be important to test the samples with other *M. bovis* antigens (e.g. PPD, LAM and MPB70/83) to directly compare the performance of EVELISA with those of reported antigens.

Conclusion

The findings presented above indicate that EVELISA could detect antibodies against *M. bovis*. However, characterization of immunodominant antigens in the ethanol extract and testing a larger number of samples for validation of the results are required. This study suggests that EVELISA can form a basis for

development of a sensitive and specific test for bTB in deer.

Abbreviations

bTB: Bovine tuberculosis; JD: Johne's disease; *M. bovis*: *Mycobacterium bovis*; MAP: *Mycobacterium avium* ssp. *paratuberculosis*; ELISA: Enzyme linked immunosorbent assay; EVELISA: Ethanol Vortex enzyme linked immunosorbent assay; TST: Tuberculin skin test; PPD: Purified protein derivatives; LAM: Lipoarabinomannan; MAPIA: Multi-antigen print immune-assay; RT: Rapid test; DPP: Dual path platform.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SE conceived the study, carried out the statistics and designed the experiments. AW, REJ conducted the experiments. AW drafted the final manuscript with the help of SE. CM, JFTG, WRW, and JPB helped in designing the experiments and provided samples – reagents for the study. All authors read and approved the final manuscript.

Authors' information

AW was a graduate student (when the study was conducted) and SE an Associate Professor in the Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee Institute of Agriculture, Knoxville, Tennessee. REJ is a Veterinary Student at the College of Veterinary Medicine, University of Tennessee, Knoxville. CGM and JFTG are senior researchers at AgResearch Invermay, New Zealand. Both, WRW is a Veterinary Medical Officer and JPB is a Research Microbiologist at USDA, Ames, IA.

Acknowledgements

The work was supported by the following grants to SE: the New Zealand Animal Health Board, University of Tennessee Research Foundation Technology Maturation Grants, University of Tennessee M-CERV seed grant; and graduate research assistantship to AW by the National Institute of Mathematical and Biological Synthesis. Portions of this work were also supported by the USDA-Agricultural Research Service.

Author details

¹Department of Forestry, Wildlife and Fisheries, Center for Wildlife Health, University of Tennessee Institute of Agriculture, Knoxville, Tennessee 37996, USA. ²AgResearch Invermay, P.O. Box 50034, Mosgiel, New Zealand. ³Disease Research Laboratory, University of Otago, Dunedin, New Zealand. ⁴Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, USDA Agricultural Research Service, Ames, Iowa 50010, USA.

Received: 8 August 2013 Accepted: 6 December 2013

Published: 17 December 2013

References

- de Lisle GW, Mackintosh CG, Bengis RG: **Mycobacterium bovis in free-living and captive wildlife, including farmed deer.** *Rev Sci Tech* 2001, **20**(1):86–111.
- Wadhwa A, Hickling GJ, Eda S: **Opportunities for improved serodiagnosis of human tuberculosis, bovine tuberculosis, and paratuberculosis.** *Vet Med Int* 2012, **2012**:674238.
- Neill SD, Pollock JM, Bryson DB, Hanna J: **Pathogenesis of Mycobacterium bovis infection in cattle.** *Vet Microbiol* 1994, **40**(1–2):41–52.
- Griffin JF, Buchan GS: **Aetiology, pathogenesis and diagnosis of Mycobacterium bovis in deer.** *Vet Microbiol* 1994, **40**(1–2):193–205.
- Michel AL, Muller B, van Helden PD: **Mycobacterium bovis at the animal-human interface: a problem, or not?** *Vet Microbiol* 2010, **140**(3–4):371–381.
- Buddle BM, Livingstone PG, de Lisle GW: **Advances in ante-mortem diagnosis of tuberculosis in cattle.** *N Z Vet J* 2009, **57**(4):173–180.
- Thoen CO, Steele JH, Gilsdorf MJ: **Epidemiology of Mycobacterium Bovis, in Mycobacterium Bovis Infection in Animals and Humans.** Secondth edition. Oxford, UK: Blackwell Publishing Ltd; 2008.
- Neill SD, Bryson DG, Pollock JM: **Pathogenesis of tuberculosis in cattle.** *Tuberculosis (Edinb)* 2001, **81**(1–2):79–86.
- O'Reilly LM, Daborn CJ: **The epidemiology of Mycobacterium bovis infections in animals and man: a review.** *Tuber Lung Dis* 1995, **76**(Suppl 1):1–46.
- Schiller I, Oesch B, Vordermeier HM, Palmer MV, Harris BN, Orloski KA, Buddle BM, Thacker TC, Lyashchenko KP, Waters WR: **Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication.** *Transbound Emerg Dis* 2010, **57**(4):205–220.
- Mackintosh CG, Littlejohn RP, Thompson BR: **Improving the tuberculin test in red deer (Cervus elaphus).** In *Deer Branch N Z Vet Assoc. 2007.* 2007:83–86.
- Waters WR, Stevens GE, Schoenbaum MA, Orloski KA, Robbe-Austerman S, Harris NB, Hall SM, Thomsen BV, Wilson AJ, Brannian RE, et al: **Bovine tuberculosis in a nebraska herd of farmed elk and fallow deer: a failure of the tuberculin skin test and opportunities for serodiagnosis.** *Vet Med Int* 2011, **2011**:953985.
- de Lisle GW, Bengis RG, Schmitt SM, O'Brien DJ: **Tuberculosis in free-ranging wildlife: detection, diagnosis and management.** *Rev Sci Tech* 2002, **21**(2):317–334.
- de la Rua-Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen KH, Clifton-Hadley RS: **Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques.** *Res Vet Sci* 2006, **81**(2):190–210.
- Queiros J, Alvarez J, Carta T, Mateos A, Ortiz JA, Fernandez-de-Miera IG, Martin-Hernando MP, Gortazar C: **Unexpected high responses to tuberculin skin-test in farmed red deer: Implications for tuberculosis control.** *Prev Vet Med* 2012, **104**(3–4):327–334.
- Palmer MV, Waters WR, Whipple DL, Slaughter RE, Jones SL: **Evaluation of an in vitro blood-based assay to detect production of interferon-gamma by Mycobacterium bovis-infected white-tailed deer (Odocoileus virginianus).** *J Vet Diagn Invest* 2004, **16**(1):17–21.
- Waters WR, Palmer MV, Thacker TC, Orloski K, Nol P, Harrington NP, Olsen SC, Nonnecke BJ: **Blood culture and stimulation conditions for the diagnosis of tuberculosis in cervids by the Cervigam assay.** *Vet Rec* 2008, **162**(7):203–208.
- Lyashchenko KP, Greenwald R, Esfandiari J, Chambers MA, Vicente J, Gortazar C, Santos N, Correia-Neves M, Buddle BM, Jackson R, et al: **Animal-side serologic assay for rapid detection of Mycobacterium bovis infection in multiple species of free-ranging wildlife.** *Vet Microbiol* 2008, **132**(3–4):283–292.
- Grange JM, Gibson J, Nassau E, Kardjito T: **Enzyme-linked immunosorbent assay (ELISA): a study of antibodies to Mycobacterium tuberculosis in the IgG, IgA and IgM classes in tuberculosis, sarcoidosis and Crohn's disease.** *Tubercle* 1980, **61**(3):145–152.
- Plackett P, Ripper J, Corner LA, Small K, de Witte K, Melville L, Hides S, Wood PR: **An ELISA for the detection of anergic tuberculous cattle.** *Aust Vet J* 1989, **66**(1):15–19.
- Hanna J, Neill SD, O'Brien JJ: **Use of PPD and phosphatide antigens in an ELISA to detect the serological response in experimental bovine tuberculosis.** *Res Vet Sci* 1989, **47**(1):43–47.
- Sugden EA, Stilwell K, Rohonczy EB, Martineau P: **Competitive and indirect enzyme-linked immunosorbent assays for Mycobacterium bovis infections based on MPB70 and lipoarabinomannan antigens.** *Can J Vet Res* 1997, **61**(1):8–14.
- Vordermeier HM, Whelan A, Cockle PJ, Farrant L, Palmer N, Hewinson RG: **Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle.** *Clin Diagn Lab Immunol* 2001, **8**(3):571–578.
- Wiker HG: **MPB70 and MPB83—major antigens of Mycobacterium bovis.** *Scand J Immunol* 2009, **69**(6):492–499.
- Griffin JF, Nagai S, Buchan GS: **Tuberculosis in domesticated red deer: comparison of purified protein derivative and the specific protein MPB70 for in vitro diagnosis.** *Res Vet Sci* 1991, **50**(3):279–285.
- Waters WR, Palmer MV, Thacker TC, Bannantine JP, Vordermeier HM, Hewinson RG, Greenwald R, Esfandiari J, McNair J, Pollock JM, et al: **Early antibody responses to experimental Mycobacterium bovis infection of cattle.** *Clin Vaccine Immunol* 2006, **13**(6):648–654.
- Lyashchenko KP, Greenwald R, Esfandiari J, O'Brien DJ, Schmitt SM, Palmer MV, Waters WR: **Rapid detection of serum antibody by dual-path platform VetTB assay in white-tailed deer infected with Mycobacterium bovis.** *Clin Vaccine Immunol* 2013, **20**(6):907–911.
- Buddle BM, Wilson T, Denis M, Greenwald R, Esfandiari J, Lyashchenko KP, Liggett S, Mackintosh CG: **Sensitivity, specificity, and confounding factors of novel serological tests used for the rapid diagnosis of bovine**

- tuberculosis in farmed red deer (*Cervus elaphus*). *Clin Vaccine Immunol* 2010, **17**(4):626–630.
29. Eda S, Elliott B, Scott MC, Waters WR, Bannantine JP, Whitlock RH, Speer CA: **New method of serological testing for *Mycobacterium avium* subsp. paratuberculosis (Johne's disease) by flow cytometry.** *Foodborne Pathog Dis* 2005, **2**(3):250–262.
 30. Eda S, Bannantine JP, Waters WR, Mori Y, Whitlock RH, Scott MC, Speer CA: **A highly sensitive and subspecies-specific surface antigen enzyme-linked immunosorbent assay for diagnosis of Johne's disease.** *Clin Vaccine Immunol* 2006, **13**(8):837–844.
 31. Speer CA, Scott MC, Bannantine JP, Waters WR, Mori Y, Whitlock RH, Eda S: **A novel enzyme-linked immunosorbent assay for diagnosis of *Mycobacterium avium* subsp. paratuberculosis infections (Johne's Disease) in cattle.** *Clin Vaccine Immunol* 2006, **13**(5):535–540.
 32. Scott MC, Bannantine JP, Kaneko Y, Branscum AJ, Whitlock RH, Mori Y, Speer CA, Eda S: **Absorbed EVELISA: a diagnostic test with improved specificity for Johne's disease in cattle.** *Foodborne Pathog Dis* 2010, **7**(11):1291–1296.
 33. Mackintosh CG, Labes RE, Thompson BR, Clark RG, de Lisle GW, Johnstone PD, Griffin JF: **Efficacy, immune responses and side-effects of vaccines against Johne's disease in young red deer (*Cervus elaphus*) experimentally challenged with *Mycobacterium avium* subsp. paratuberculosis.** *N Z Vet J* 2008, **56**(1):1–9.
 34. Mackintosh CG, Qureshi T, Waldrup K, Labes RE, Dodds KG, Griffin JF: **Genetic resistance to experimental infection with *Mycobacterium bovis* in red deer (*Cervus elaphus*).** *Infect Immun* 2000, **68**(3):1620–1625.
 35. Griffin JF, Chinn DN, Rodgers CR: **Diagnostic strategies and outcomes on three New Zealand deer farms with severe outbreaks of bovine tuberculosis.** *Tuberculosis (Edinb)* 2004, **84**(5):293–302.
 36. Mackintosh C, Clark G, Tolentino B, Liggett S, De Lisle G, Griffin F: **Longitudinal Pathogenesis Study of Young Red Deer (*Cervus elaphus*) after Experimental Challenge with *Mycobacterium avium* subsp. paratuberculosis (MAP).** *Vet Med Int* 2012, **2012**:931948.
 37. Greiner M, Sohr D, Gobel P: **A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests.** *J Immunol Methods* 1995, **185**(1):123–132.
 38. Waters WR, Palmer MV, Whipple DL: ***Mycobacterium bovis*-infected white-tailed deer (*Odocoileus virginianus*): detection of immunoglobulin specific to crude mycobacterial antigens by ELISA.** *J Vet Diagn Invest* 2002, **14**(6):470–475.
 39. Waters WR, Palmer MV, Bannantine JP, Whipple DL, Greenwald R, Esfandiari J, Andersen P, McNair J, Pollock JM, Lyashchenko KP: **Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*.** *Clin Diagn Lab Immunol* 2004, **11**(5):849–855.
 40. Waters WR, Palmer MV, Bannantine JP, Greenwald R, Esfandiari J, Andersen P, McNair J, Pollock JM, Lyashchenko KP: **Antibody responses in reindeer (*Rangifer tarandus*) infected with *Mycobacterium bovis*.** *Clin Diagn Lab Immunol* 2005, **12**(6):727–735.
 41. Greenwald R, Lyashchenko O, Esfandiari J, Miller M, Mikota S, Olsen JH, Ball R, Dumonceaux G, Schmitt D, Moller T, et al: **Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants.** *Clin Vaccine Immunol* 2009, **16**(5):605–612.
 42. Harrington NP, Surujballi OP, Prescott JF, Duncan JR, Waters WR, Lyashchenko K, Greenwald R: **Antibody responses of cervids (*Cervus elaphus*) following experimental *Mycobacterium bovis* infection and the implications for immunodiagnosis.** *Clin Vaccine Immunol* 2008, **15**(11):1650–1658.
 43. Boadella M, Barasona JA, Diaz-Sanchez S, Lyashchenko KP, Greenwald R, Esfandiari J, Gortazar C: **Performance of immunochromatographic and ELISA tests for detecting fallow deer infected with *Mycobacterium bovis*.** *Prev Vet Med* 2012, **104**(1–2):160–164.
 44. Wadhwa A, Bannantine JP, Byrem TM, Stein TL, Saxton AM, Speer CA, Eda S: **Optimization of serum EVELISA for milk testing of Johne's disease.** *Foodborne Pathog Dis* 2012, **9**(8):749–754.

doi:10.1186/1746-6148-9-256

Cite this article as: Wadhwa et al.: Use of ethanol extract of *Mycobacterium bovis* for detection of specific antibodies in sera of farmed red deer (*Cervus elaphus*) with bovine tuberculosis. *BMC Veterinary Research* 2013 **9**:256.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

