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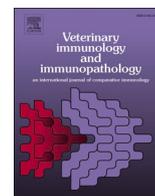
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## Differential detection of IgM and IgG antibodies to chimeric antigens in bovine tuberculosis

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### ABSTRACT

Recent studies have suggested the potential of innovative serologic tests for accurate and rapid detection of bovine tuberculosis (bTB). Dual Path Platform (DPP) technology has been used to develop rapid animal-side antibody tests for *Mycobacterium bovis* infection in a range of livestock and wildlife host species. The present study evaluated diagnostic performance of DPP BovidTB IgM/IgG assay designed for differential detection of bovine IgM and IgG antibodies against two chimeric antigens, DID38 and TBf2, respectively, using 662 well-characterized serum samples from *M. bovis*-infected and bTB-free cattle collected in the United States, Great Britain, France, and South Africa. Test sensitivity and specificity ranged from 71% to 100% and from 95% to 100%, respectively, depending on the country, with overall accuracy of 83%. No significant risk of cross-reactivity with serum samples from cattle infected with most relevant species of mycobacteria other than *M. bovis* was found. The DPP BovidTB IgM/IgG assay may be suitable for use in multi-test algorithms to improve current strategies for bTB surveillance.

### 1. Introduction

Bovine tuberculosis (bTB) caused by pathogens of the *Mycobacterium tuberculosis* complex, predominantly *M. bovis* or *M. caprae* but also *M. tuberculosis*, remains a major zoonotic disease affecting multiple livestock and wildlife species worldwide (Gormley and Corner, 2013; Palmer, 2013). The current ante-mortem tests for bTB relying on cell-mediated immune responses, such as the tuberculin skin test (TST) or *in vitro* interferon-gamma release assay (IGRA), suffer from sub-optimal diagnostic accuracy, variable performance in different geographic areas, and unaffordable cost for low-income countries (Buddle et al., 2009; Schiller et al., 2010; Bass et al., 2013). While the search for novel biomarkers of cell-mediated responses continues (Palmer et al., 2020; Steinbach et al., 2021), numerous studies have

demonstrated the utility of emerging serologic assays for rapid detection of *M. bovis* infection in a range of domesticated animals and wildlife hosts (Dean et al., 2009; Waters et al., 2017; Lyashchenko et al., 2018; Miller et al., 2019; Bernitz et al., 2021; Thomas et al., 2021). Screening of over 100 recombinant protein candidates for serodiagnostic use by multi-antigen print immunoassay and Dual Path Platform (DPP) technology identified a set of novel targets for the antibody responses in *M. bovis* infection, some of which were integrated in a series of multi-epitope fusion polyproteins designed for improved serodiagnostics (Lyashchenko et al., 2017b, 2021b). The antibody assays have been proposed for implementation in bTB surveillance as stand-alone diagnostic tools or as ancillary tests applied in conjunction with TST or IGRA to enable highly sensitive multi-test algorithms (Coat et al., 2008; Casal et al., 2014; Waters et al., 2017; Sridhara et al., 2022).

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In the present study, we evaluated performance of the DPP BovidTB IgM/IgG assay using 662 well-characterized serum samples collected from *M. bovis*-infected and bTB-free cattle in four different countries. The design of this newly developed combo antibody test was informed by recent studies (Lyashchenko et al., 2021b; Sridhara et al., 2022) employing two polypeptide fusion antigens, DID38 and TBf2, for differential detection of bovine IgM and IgG antibodies, respectively. A rapid and accurate serologic test for bTB would provide another useful tool for screening of cattle herds.

## 2. Materials and methods

### 2.1. Animals and test specimens

Serum samples were collected from cattle in herds infected with *M. bovis* (n = 363) and from bTB-free herds (n = 299) in the United States (US), Great Britain (GB), France, and South Africa. Diagnosis was confirmed post-mortem by *M. bovis* culture isolation, as previously described (Palmer et al., 2006; Waters et al., 2017). The samples collected from cattle in GB and South Africa were tested by DPP BovidTB IgM/IgG assay in the respective countries, whereas the samples collected in the US and France were tested in the US. A subset of 72 GB samples was tested repeatedly in the US to assess reproducibility.

To evaluate test specificity, serum samples obtained from the following experimentally treated groups of calves were used: 1) intratonsillar-inoculated with  $\sim 10^{10}$  CFU *M. avium* subsp. *avium*, strain 3988, a bovine isolate (n = 8), 2) intratonsillar-inoculated with  $\sim 10^8$  CFU *M. avium* subsp. *paratuberculosis* (MAP), strain K-10 (n = 7), 3) intratonsillar-inoculated with  $\sim 10^8$  CFU *M. kansasii*, strain 03-6391, a bovine isolate from a pyogranulomatous lymph node (n = 8), and 4) subcutaneously injected with 0.5 ml heat-inactivated whole-cell MAP vaccine Mycopar® (Ford Dodge Animal Health, IA, US) (n = 10). The infection or vaccination experiments were initiated on Holstein steers at  $\sim 4$ –6 months of age obtained from known bTB-free herds in Iowa and housed in a biosafety level-2 facility at the National Animal Disease Center, Ames, IA, according to Institutional Biosafety and Animal Care and Use Committee guidelines, as described previously (Palmer et al., 2002; Waters et al., 2004, 2006; Stabel et al., 2013). The serum samples were collected from the above animal groups 6–8 months post-inoculation. In addition, specimens were obtained from 29 cattle subcutaneously injected with 1 ml heat-inactivated whole-cell MAP vaccine Gudair® (Virbac Ltd, UK) in GB as described (Middleton et al., 2021). All serum samples were kept frozen at  $-20$  °C until use in antibody assays.

### 2.2. Antibody detection

Bovine sera were tested with DPP BovidTB IgM/IgG assay following the test procedure established previously (Lyashchenko et al., 2017a, 2021a). The combo assay uses colloidal gold nanoparticles functionalized with goat anti-bovine IgM antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) or protein A/G (BioVision, Milpitas, CA, USA) for differential detection of IgM and IgG antibodies, respectively, on two separate test strips with independent sample flows. Test line intensity was measured by an optical reader instrument in relative light units (RLU). Cutoff values for differential detection of IgM and IgG antibodies were established by Receiver Operating Characteristic (ROC) curve analyses using Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) as previously described (Lyashchenko et al., 2021b). Based on a signal-to-cutoff ratio (S/CO) calculated for each antibody isotype, test results were interpreted as antibody positive at S/CO  $\geq 1.0$  or antibody negative at S/CO  $< 1.0$ .

### 2.3. Data analysis

Test sensitivity and specificity were determined against the gold

standard of *M. bovis* culture with the 95% confidence intervals (95% CI) and Kappa value calculated using Vassar Stats software (vassarstats.net). Sensitivity was estimated as the proportion of animals with confirmed *M. bovis* infection that tested antibody positive. Specificity was calculated as the proportion of uninfected animals that tested antibody negative. Diagnostic accuracy was determined as the proportion of true results, including both antibody positive and antibody negative, in all tested populations.

## 3. Results and discussion

Prompted by recent findings (Sridhara et al., 2022), we developed DPP BovidTB IgM/IgG assay, a new-generation rapid combo test for simultaneous and differential detection of IgM and IgG antibodies to DID38 and TBf2 polyproteins, respectively. Both fusion antigens include immunodominant MPB70 and MPB83 proteins of *M. bovis*, whereas TBf2 also integrates full sequences of two additional proteins, CFP10 and Rv2650c (Lyashchenko et al., 2021b). The use of two distinct chimeric antigens for detection of the above isotypes is expected to cover a broader spectrum of variable antibody reactivity patterns in cattle populations to maximize test sensitivity, as has been previously suggested (Lyashchenko et al., 2017b, 2021b).

The goal of the present study was to evaluate diagnostic performance of DPP BovidTB IgM/IgG assay. We tested 363 serum samples collected from *M. bovis*-infected cattle and 299 samples from bTB-free herds in the US, GB, France, and South Africa. Overall test sensitivity and specificity estimates were 73.3% and 95.3%, respectively (Table 1). When analyzed per country, sensitivity ranged from 70.7% in the US to 100% in France. Specificity was less variable across the countries ranging from 94.9% in the US to 100% in South Africa. Overall diagnostic accuracy was 83.2%.

Of the 75 samples collected from *M. bovis*-infected cattle in GB and tested at APHA (Table 1), 72 were available for an independent study performed in a blindly coded fashion by Chembio laboratory in the US. At both sites, DPP BovidTB IgM/IgG assay produced 57 positive results (79.2%), demonstrating a perfect concordance (Kappa = 1.000). The results support highly reproducible test performance when used by different operators at different locations.

The analytical specificity of DPP BovidTB IgM/IgG assay was additionally tested with serum samples collected from 62 calves inoculated either with *M. avium*, *M. kansasii*, or MAP, or with two commercial MAP vaccines. As shown in Table 2, no IgM or IgG antibody was detected in any of potentially cross-reactive samples from 23 cattle experimentally infected with mycobacteria other than *M. bovis*, predicting a low risk of false-positive results due to possible exposure to non-bTB environmental or pathogenic mycobacteria. Among 39 samples from MAP-vaccinated cattle, we found two IgM positive results (5.1%) among those receiving Gudair vaccine. This makes the overall false-positivity rate of

**Table 1**

Diagnostic performance of DPP BovidTB IgM/IgG assay in cattle from different geographic areas.

Country	Sensitivity		Specificity	
	n/N <sup>a</sup>	% (95% CI)	n/N <sup>a</sup>	% (95% CI)
United States	193/273	70.7 (64.8–75.9)	223/235	94.9 (91.0–97.2)
Great Britain	60/75	80.0 (68.9–88.0)	52/54	96.3 (86.2–99.4)
France	8/8	100 <sup>b</sup>	NT <sup>c</sup>	
South Africa	5/7	71.4 <sup>b</sup>	10/10	100.0 <sup>b</sup>
<b>Total</b>	<b>266/363</b>	<b>73.3 (68.4–77.7)</b>	<b>285/299</b>	<b>95.3 (92.1–97.3)</b>

<sup>a</sup> N, number of animals tested; n, number of true results defined by disease status;

<sup>b</sup> 95% CI was not calculated due to relatively small animal groups;

<sup>c</sup> NT, not tested.

**Table 2**

Analytical specificity of DPP BovidTB IgM/IgG assay evaluated with sera from cattle vaccinated for MAP or inoculated with mycobacteria other than *M. bovis*.

Animal group	Antibody reactivity (n/N <sup>a</sup> )		
	IgM	IgG	IgM and/or IgG
<i>M. avium</i> -inoculated	0/8	0/8	0/8
<i>M. kansasii</i> -inoculated	0/8	0/8	0/8
MAP-inoculated	0/7	0/7	0/7
MAP-vaccinated <sup>b</sup>	2/39	0/39	2/39

MAP, *M. avium* subsp. paratuberculosis.

<sup>a</sup> N, number of animals tested; n, number of antibody reactive results;

<sup>b</sup> Animal group consisted of 10 cattle that received Mycopar vaccine and 29 cattle that received Gudair vaccine;

3.2% observed in this part of test evaluation (Table 2) consistent with the specificity of 95.3% estimated with samples from bTB-free herds (Table 1), thus suggesting a low risk of possible cross-reactivity with the three relevant species of other mycobacteria tested.

The present study used larger numbers of specimens from the US and GB than those available from France and South Africa. The GB data set showed a higher diagnostic accuracy of DPP BovidTB IgM/IgG assay (86.8%; 95% CI: 79.5–91.9%) as compared to that obtained with the US samples (81.9%; 95% CI: 78.2–85.1%), suggesting a more balanced relationship between test sensitivity and specificity (Table 1) to better discriminate between *M. bovis*-infected and bTB-free animals. The higher sensitivity found with GB samples may be partly attributed to the fact that all 75 *M. bovis*-infected cattle were TST reactors and were bled 10–30 days post-TST administration, whereas in the US, 31/273 cattle diagnosed with bTB were TST non-reactors (11.4%) and serum samples were collected prior to or within 3 days of tuberculin injection.

Fig. 1 shows semi-quantitative serological results of the GB cattle testing to provide an example illustrating relative magnitudes of IgM and IgG antibody responses. The IgM levels appeared to be generally lower than IgG values. However, among 45/75 (60%) *M. bovis*-infected cattle that developed IgM antibodies, 6 animals produced IgM antibodies in the absence of detectable IgG antibodies, thereby contributing an extra 8% to the total combination test sensitivity estimate of 80% (Table 1). Five out of eight cattle in the bTB group from France showed IgM antibody, and all of them tested IgG antibody positive. In the group of *M. bovis*-infected cattle from South Africa, 4/7 were IgM positive, 4/7 were IgG positive, and 5/7 were positive for IgM and/or IgG antibody, implying that the sensitivity of DPP BovidTB IgM/IgG assay benefits from the combined detection of IgM and IgG antibodies. These findings are in line with our recent report describing complementary IgM antibody reactivity rates ranging from 4.1% in the US to 23.5% in Spain (Sridhara et al., 2022), presumably reflecting country-to-country differences in veterinary practices and cattle populations with variably predominant bTB stages.

The idea of supplementing IgG antibody tests with IgM detection is not new in the field of serodiagnosis of infectious diseases. In humans, this approach has been extensively explored for earlier detection of acute HIV infection, syphilis, leptospirosis, Zika fever, Lyme disease, COVID-19, and many others (Alexander, 2016; Branda and Steere, 2021; Satyaputra et al., 2021). Experiments in cattle inoculated with *M. bovis* have suggested added diagnostic value of IgM antibody detection, particularly at early stages of infection (Waters et al., 2006; Lyashchenko et al., 2017a). Furthermore, it has been shown that IgM responses in bTB can be elicited and/or boosted by TST much faster than IgG responses (Lyashchenko et al., 2017a), presumably due to a swift activation of circulating and tissue-resident IgM+ memory B cells elicited by intradermal tuberculin injection (Lyashchenko et al., 2020). The booster effect may facilitate a further increase in test sensitivity through earlier serodiagnosis relying on IgM responses. Further, use of a rapid test capable of differentiating the two antibody isotypes during herd outbreak investigations may provide useful information on early

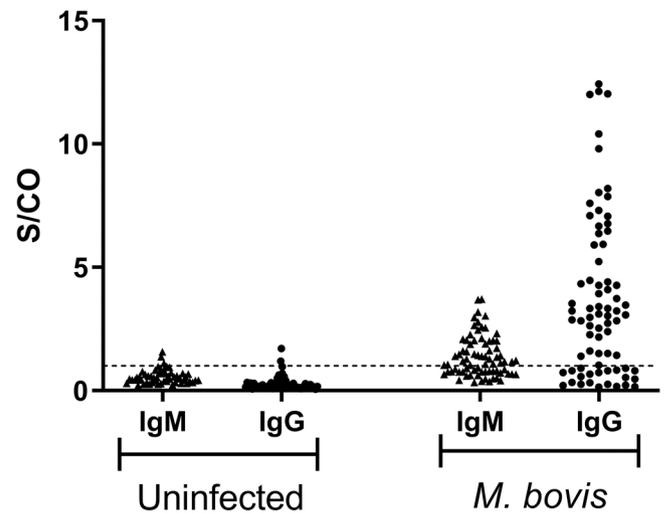


Fig. 1. IgM and IgG antibody responses measured by DPP BovidTB IgM/IgG assay in cattle with bTB in GB. Results are shown as individual values of DPP reader-generated signal-to-cutoff ratios (S/CO) for IgM antibodies (triangles) and IgG antibodies (circles) detected in *M. bovis*-infected and uninfected animals. Values above dashed line (S/CO = 1.0) indicate antibody positive results.

infection versus advanced disease predominantly associated with IgM and IgG responses, respectively (Waters et al., 2006; Lyashchenko et al., 2017a).

The diagnostic performance of the DPP BovidTB IgM/IgG assay, characterized in the present study, is consistent with earlier reports describing IgM and IgG antibodies in cattle with bTB (Waters et al., 2006, 2017; Lyashchenko et al., 2017b, 2021b). It has been proposed that serologic assays may be suitable for multi-test algorithms including TST (Coad et al., 2008; Casal et al., 2014; Sridhara et al., 2022) or used as stand-alone blood-based assays for rapid identification of *M. bovis*-infected cattle in high bTB burden areas where other ante-mortem test options may not be readily available (de la Rua-Domenech et al., 2006; Schiller et al., 2010). Multi-test algorithms including IgM and IgG antibody detection by independent DPP assays used in conjunction with TST and/or IGRA have achieved an overall sensitivity of 95–96% in bTB-affected cattle herds in the US and Spain (Sridhara et al., 2022). In addition, recent studies have demonstrated the presence of antibodies in bodily fluids other than blood (saliva, broncho-alveolar lavage, extracts from lungs or lymph nodes), some of which appear to contain comparable levels of circulating IgG antibodies to those found in matching serum or plasma samples from animals infected with *M. bovis* (Lyashchenko et al., 2021a). Flexibility in sample usage offers practical field applications, particularly for wildlife disease surveillance.

In conclusion, timely identification of bTB may benefit from implementation of rapid antibody tests based on innovative reagents and advanced formats, such as the DPP BovidTB IgM/IgG assay, which can be used in the diagnostic laboratory or, if needed, animal-side under field conditions. The present findings indicate that combined detection of IgM and IgG antibodies to chimeric antigens offers increased test sensitivity. One limitation of this work was the availability of only small numbers of well-characterized cattle samples from France and South Africa as compared to those collected in the US and GB. Future studies will validate the suggested diagnostic potential of rapid and accurate antibody assays proposed for detection of *M. bovis* infections in multiple livestock and wildlife host species using the most efficient testing strategies in bTB surveillance programs.

#### Declaration of competing interest

The authors declare no conflict of interest.

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