Evaluation of the Dual Path Platform (DPP) VetTB assay for the detection of Mycobacterium bovis infection in badgers

Roland T. Ashford, Paul Anderson, Laura Waring, Dipesh Davé, Freya Smith, Richard J. Delahay, Eamonn Gormley, Mark A. Chambers, Jason Sawyer, Sandrine Lesellier

ARTICLE INFO
Keywords:
Badgers
Bovine tuberculosis
Diagnosis
Mycobacterium bovis
Sensitivity
Specificity

ABSTRACT
Bovine tuberculosis (bTB), caused by Mycobacterium bovis, represents a major animal health issue. In the United Kingdom and the Republic of Ireland, European badgers (Meles meles) have been shown to act as a reservoir of M. bovis infection, hindering the eradication of bTB in livestock. The availability of suitable diagnostic assays, particularly those that may be applied in a “trap-side” setting, would facilitate the implementation of a wider range of disease control strategies. Here we evaluate the Dual Path Platform (DPP) VetTB assay, a lateral-flow type test for detecting antibodies to M. bovis antigens (MPB83 and ESAT-6/CFP-10). Both serum and whole blood were evaluated as diagnostic samples. Additionally, two methods were evaluated for interpretation of test results (qualitative interpretation by eye and quantitative measurement using an optical reader). The antibody response to MPB83 detected by the DPP VetTB assay increased significantly following experimental M. bovis infection of badgers, whilst the response to ESAT-6/CFP-10 showed no significant change. In sera from TB-free captive and naturally M. bovis infected wild badgers the MPB83 response exhibited a sensitivity of 55 % by eye and quantitative reader (95 % CI: 38–71, respectively), with slightly lower specificity when read by eye (93 % compared to 98 %; 95 % CI: 85–100 and 90–100, respectively). In whole blood, the DPP VetTB assay MPB83 response exhibited a sensitivity of 65 % (95 % CI: 50–80) when interpreted by eye and 53 % (95 % CI: 36–69) using quantitative values, whilst the specificity was 94 % and 98 % respectively (95 % CI: 88–100 and 90–100). Comparison with contemporaneous diagnostic test results from putatively naturally infected and TB-free badgers demonstrated varying levels of agreement. Using sera from naturally M. bovis infected and TB-free badgers, with post mortem confirmation of disease status, the DPP VetTB assay exhibited a sensitivity of 60 % (95 % CI: 41–77) when interpreted using quantitative values (specificity 95 %; 95 % CI: 76–100), and 67 % (95 % CI: 50–84) when read by eye (specificity 95 %; 95 % CI: 86–100). Further work is required to robustly characterize the DPP VetTB assay’s performance in a wider selection of samples, and in the practical and epidemiological contexts in which it may be applied.

1. Introduction
Bovine tuberculosis (bTB), caused by Mycobacterium bovis, represents an on-going challenge for the farming industry in parts of Europe, North America and New Zealand (Gormley and Corner, 2018), and is recognised as a re-emerging animal- and public-health issue in a number of low and middle income countries (Olea-Popelka et al., 2017). In those countries that have implemented statutory control programmes, the eradication of bTB in livestock may be hindered by the existence of a reservoir of infection in wildlife (Gormley and Corner, 2018). In the United Kingdom and the Republic of Ireland, the European badger (Meles meles) has been shown to act as such a reservoir of infection, and is implicated in the transmission of infection to cattle (Palmer, 2013). One of the primary challenges in tackling M. bovis infection in wildlife is the lack of sufficiently sensitive and specific diagnostic tests, which may be used to assess disease prevalence, identify
infected individuals or populations for control purposes or to monitor the success of intervention strategies (Maas et al., 2013).

The “gold-standard” for TB diagnosis in badgers is a detailed post mortem examination and culture of tissues for viable *M. bovis*. Studies of naturally infected badger populations in both the United Kingdom and the Republic of Ireland have demonstrated that a significant proportion of infected animals do not have macroscopic lesions, so histological examination and bacteriological culture of tissues are essential in order to maximise the sensitivity of diagnosis (Grawshaw et al., 2008; Murphy et al., 2010). Infection can also be diagnosed by the culture of *M. bovis* from clinical samples taken from live animals (Gallagher and Clifton-Hadley, 2000). However, this approach lacks sensitivity, in part due to the intermittent nature of detectable bacterial excretion among infected animals (Chambers et al., 2000). Nonetheless, the specificity of culture of infected animals do not have macroscopic lesions, so histological analysis to be 99.8 %, making a positive culture result highly diagnostically informative (Drewe et al., 2010). Here we report the first evaluation of the Chembio Dual-Path Platform (DPP™) VetTB assay, manufactured by Chembio Diagnostic Systems Inc., is a rapid lateral-flow type test for antibodies to TB infection in cervids and elephants. The assay detects IgG antibodies, and has two immuno-chromatographic test lines, separately detecting responses to MPB83 and ESAT-6/CFP-10 fusion protein. Data on the performance of the DPP VetTB assay have been generated for various species of deer (Lyashchenko et al., 2013), South American camelids (Lyashchenko et al., 2011), wild boar (Che’ Amat et al., 2015) and elephants (Greenwald et al., 2009; Lyashchenko et al., 2012). The performance of the assay appears to vary significantly between species, with published sensitivity estimates ranging from 59.3 to 61.5% in wild boar (*Sus scrofa*) to 71.9 % in naturally infected white-tailed deer (*Odocoileus virginianus*).

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Brief description</th>
<th>Purpose</th>
<th>Number of samples</th>
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<td>Samples from TB-free captive badgers</td>
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<td>VES</td>
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<td>BVS</td>
<td>Samples from wild badgers naturally infected with <em>M. bovis</em>, confirmed by culture</td>
<td>Estimates of sensitivity in naturally infected badgers</td>
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N/A: Not applicable; VES: Vaccine Efficacy Study; BVS: Badger Vaccine Study; BVFT: Badger Vaccination Field Trial.

1 Whole blood samples tested without freeze-thawing.
2 Samples from 12 experimentally infected badgers collected on six consecutive occasions.

A retrospective study design was employed to evaluate the diagnostic performance of the DPP VetTB assay, utilizing existing serum and whole blood samples (i.e. convenience samples, as defined in STARD guidelines; Cohen et al., 2016). Further details are given in Table 1.

2. Materials and methods

2.1. Samples from TB-free captive badgers (Weybridge)

Samples were collected from captive badgers free of *M. bovis* infection, for assessment of DPP VetTB assay specificity. These animals were originally trapped in counties of England (Essex and Suffolk) within the “low risk” area for TB incidence in the cattle population, where there is considered to be no significant reservoir of TB in wildlife (Defra, 2014). Once in captivity, these animals were tested using the parallel and serial application of a combination of existing diagnostic assays. Badgers were tested on three consecutive occasions, at approximately monthly intervals, using an interferon-gamma ELISA (Dalley et al., 2008), with all results negative. Concurrently, clinical
samples (tracheal mucous, laryngeal swabs, rectal swabs and urine) were collected for bacteriological culture to detect the presence of excreted *M. bovis* (Chambers et al., 2017 for methodological details), with all samples negative. Finally, TB-free captive badgers were subsequently used in experimental infection studies (see below), with detailed post mortem examination and bacteriological culture of tissues. Molecular typing of *M. bovis* recovered from tissues (spoligotyping and VNTR analysis, see Chambers et al., 2017) confirmed that only the strain used for challenge was present, providing further confirmation of the *M. bovis*-free status of these individuals, prior to experimental infection.

2.2. Samples from badgers experimentally infected with *M. bovis* (VES)

Samples from experimentally infected badgers were collected during a vaccine efficacy study (VES) conducted as part of a programme of work to develop an oral BCG vaccine formulation for badgers (Chambers et al., 2014). Only samples collected from non-vaccinated experimentally infected animals (n = 12) were included for analysis in the current study. Briefly, badgers were confirmed to be free of TB (as described above), prior to experimental infection via endo-bronchial installation of approximately 10^5 CFU of *M. bovis* (Chambers et al., 2017). Subsequently, blood samples were collected at approximately fortnightly intervals. Twelve weeks after experimental infection badgers were euthanased and submitted to a detailed post mortem examination of gross pathology, with bacteriological culture and histological examination of tissues (Chambers et al., 2017). All these experimentally infected animals exhibited gross pathology, confirmed by culture and histology, consistent with established *M. bovis* infection (data not shown).

2.3. Samples from wild badgers naturally infected with *M. bovis*, confirmed by culture (BVS)

Samples from culture-confirmed naturally infected badgers, for preliminary estimates of relative sensitivity, were collected during the Badger Vaccine Study (BVS), a clinical field study of the safety of intramuscular BCG vaccination (Chambers et al., 2011). Briefly, a population of badgers in a high bTB incidence area of the south-west of England were trapped regularly over a period of four years. At each capture, individuals were anaesthetised and blood and clinical samples (tracheal aspirate, urine, faeces and swabs from any wounds) for culture of viable *M. bovis*, were collected. Contemporaneous BrockTB Stat-Pak test results, conducted on sera shortly after sample collection, were available for these animals (Chambers et al., 2011). Serum and blood samples from a panel of non-vaccinated control animals with culture confirmed excretion of viable *M. bovis* in clinical samples were selected for testing by DPP VetTB assay. To confirm that freezing of whole blood samples had not had a deleterious impact on *M. bovis* specific antibodies present, they were also tested using the BrockTB Stat-Pak assay, according to the manufacturer’s protocol for serum, to enable comparison with contemporaneous results for the matched sera.

2.4. Samples from badgers in an intensively studied wild population, with existing diagnostic test results (Woodchester Park)

Additional samples for the assessment of the DPP VetTB assay were collected from an intensively studied population of badgers in Woodchester Park (WP) Gloucestershire, a high BTB prevalence area in the south-west of England (Delahay et al., 2000). As part of the ongoing study of this population, badgers are live-trapped four times per year. The infection status of each trapped individual was assessed on the basis of the BrockTB Stat-Pak serological test, interferon-gamma ELISA and bacteriological culture from clinical samples (tracheal and oesophageal aspirates, urine, faeces and swabs from any bite wounds and abscesses present). For the purposes of the current study, 251 serum and whole blood samples collected during the 2014–2015 trapping season (May–January) were tested in parallel, using the DPP VetTB assay.

2.5. Samples from naturally infected and TB-free badgers with detailed post mortem results (BVFT)

Surplus serum samples collected during the Badger Vaccination Field Trial (BVFT), to investigate the impact of oral vaccination of free-living badgers against naturally transmitted *M. bovis* infection in the Republic of Ireland (Gormley et al., 2017), were used to assess the performance of the DPP VetTB assay. Detailed post mortem examination results, with bacteriological culture for *M. bovis* and histological examination of tissues, were available for 51 animals. Twenty-one animals were identified as TB negative on this basis and DPP VetTB assay results from these badgers were compared to those from 30 individuals with culture and/or histologically confirmed *M. bovis* infection. Contemporaneous BrockTB Stat-Pak results were also available for these animals (Gormley et al., 2017).

2.6. Sample collection and storage

In all cases, blood for serological testing was collected from the jugular vein of anaesthetised badgers. In the case of serum, blood was collected using either plain or serum separator (SST) vacutainer tubes (BD, Plymouth, UK). Following transfer to the laboratory, tubes were centrifuged (2600 g for 10 min) and serum removed from the clotted sample. In the case of whole blood, samples were collected using heparinised vacutainer tubes (BD, Plymouth, UK).

Serum and whole blood samples were aliquoted after collection into multiple small volume single-use aliquots (100 μL–500 μL) for subsequent testing, to avoid the need for multiple freeze-thaw cycles. In the case of whole blood collected from Woodchester Park badgers, and additional whole blood from captive TB-free badgers for assessment of specificity, samples were tested directly after collection (≤6 h), without freeze-thawing. Frozen serum and whole blood aliquots were stored at −80 °C.

2.7. DPP VetTB assay protocol

The DPP VetTB assay was performed as described by the manufacturer (DPP® VetTB Assay for Cervids, Chembio Diagnostic Systems Inc., Medford, NY, USA), with a number of minor modifications, primarily relating to the volume of sample used. Preliminary data (not shown) indicated that the serum volume recommended by the manufacturer (5 μL) resulted in sub-optimal sensitivity, relative to the BrockTB Stat-Pak. Consequently, samples from experimentally infected badgers (VES), and from captive TB-free badgers (Weybridge), were used to compare the performance of the assay with three different serum volumes (5 μL, 10 μL and 30 μL, see Supplementary Material). All subsequent evaluation work utilised a serum sample volume of 30 μL, with an additional drop of sample diluent added to the sample + buffer well (well 1) on the assay device (see Supplementary Material). Test results were read 15–20 min after addition of buffer to the buffer well (well 2).

The protocol used for whole blood was as described by the manufacturer for the DPP® VetTB Assay for Elephants, using 10 μL of whole blood. Some degree of clotting and/or haemolysis was frequently observed following freeze-thawing of whole blood samples (Weybridge, BVS and VES animals), despite the addition of heparin during collection. Consequently, samples were thoroughly mixed following thawing, prior to the removal of the required volume for testing.

The DPP VetTB assay has two test lines (plus a control line), detecting antibodies to MPB83 (Line 1) and ESAT-6/CFP-10 fusion protein (Line 2). Assay results were read both “by eye” (based upon interpretation by the operator performing the test) and using an optical
reading device supplied by the test manufacturer (Opticon DPP test reader, Chembio Diagnostic Systems Inc, USA), which measures relative light unit (RLU) values. Results read by eye were recorded as positive or negative, with a further semi-quantitative scoring of the strength of the antigen specific response (see Supplementary Material). Only assays with a valid control line result were included in subsequent analyses.

2.8. Data analysis

RLU values were analysed using non-parametric statistics (Friedman’s test, Spearman’s correlation and two-sample Kolmogorov-Smirnov test). Data were log(x + 1)-transformed where required (e.g. for parametric statistical analysis by one-way ANOVA and repeated measures two-way ANOVA). The significance of differences in the proportion of tests positive/negative was assessed using Fisher’s exact test. Sensitivity and specificity of quantitative data, relative to existing diagnostic results, were estimated by receiver operating characteristic (ROC) analysis (Greiner et al., 2000). Working cut-offs for RLU values were derived from ROC analysis, by selecting the threshold with the highest likelihood ratio. Agreement between diagnostic tests was quantified using Cohen’s kappa statistic, with interpretations as described by Dohoo et al. (2009). Statistical tests were implemented in either GraphPad Prism version 7.3 (GraphPad Software, La Jolla, CA, USA) or Minitab version 17 (Minitab Inc., PA, USA).

3. Results

3.1. Relative performance of diagnostic antigens and antibody kinetics following experimental infection (VES)

The performance of the VetTB DPP assay was initially evaluated using samples collected at five time-points following experimental infection (VES), to assess the relative diagnostic performance of the antigenic targets included in test lines one and two (MPB83 and ESAT-6/CFP-10 respectively). Line 1 (MPB83) specific antibody levels increased significantly in serum following experimental infection (Fig. 1a; Friedman test, F = 44.18, P < 0.0001). Conversely, Line 2 (ESAT-6/CFP-10) specific antibody levels in serum showed no significant increase over time (Fig. 1b; Friedman test, F = 10.63, P > 0.05). The same pattern of change in antigen specific antibodies was observed in whole blood samples collected during the same study (Fig. 1c and d). Line 1 (MPB83) specific antibody levels measured by the DPP VetTB assay increased significantly in whole blood following experimental infection, whilst no significant change was observed in Line 2 (ESAT-6/CFP-10) specific antibodies (Friedman test, F = 49.35, P < 0.0001 and F = 4.69, P > 0.05 for Line 1 and Line 2 respectively).

3.2. Estimates of sensitivity and specificity in culture confirmed naturally infected (BVS) and TB-free captive (Weybridge) badgers

Data from BVS and Weybridge badgers were used to generate preliminary estimates of sensitivity and specificity for the VetTB DPP assay, and to derive working cut-off thresholds for the quantitative reader RLU values. Summarised relative sensitivity and specificity estimates are shown in Table 2. In this panel of sera, Line 1 of the VetTB DPP assay exhibited a sensitivity of 55.3 % (95 % CI: 38.3–71.4) at a specificity of 98.1 % (95 % CI: 89.9–100.0) (Fig. 2). Consistent with responses observed in experimentally infected badgers, Line 2 results for the same samples were less diagnostically informative, with a maximum specificity of 66.0 % (95 % CI: 51.7–78.5) achieved (data not shown). The areas under the ROC curves for Line 1 and Line 2 were 0.96 (95 % CI: 0.92–1.00) and 0.66 (95 % CI: 0.54 to 0.78), respectively. When read by eye, the sensitivity of the Line 1 response in sera was 53.3 % (95 % CI: 39.5–71.1) at a specificity of 92.5 % (95 % CI: 85.3–99.6). Contemporaneous diagnostic test data for the BVS serum samples demonstrated that the BrockTB Stat-Pak assay exhibited a sensitivity of 65.0 % (95 % CI: 50.2–79.8).

The performance of the DPP VetTB assay in whole blood samples collected from the same animals was very similar, with a sensitivity of 52.5 % (95 % CI: 36.1–68.5) at a specificity of 98.1 % (95 % CI: 86.9–100.0) in Line 1 (Fig. 2). As observed in sera from the same animals, Line 2 results for whole blood were less diagnostically informative, with the maximum specificity achieved being 62.3 % (95 % CI: 47.9–75.2) (data not shown). The area under the ROC curve for Line 1 and Line 2 was 0.78 (95 % CI: 0.68–0.89) and 0.68 (95 % CI: 0.57–0.80), respectively. When read by eye, the sensitivity of the Line 1 response in whole blood was 65.0 % (95 % CI: 50.2–79.8) at a specificity of 94.3 % (95 % CI: 88.1–100.0). The BrockTB Stat-Pak assay performed on the BVS whole blood samples exhibited a sensitivity of 62.5 % (95 % CI: 47.5–77.5).

The specificity of the DPP VetTB assay was subsequently further investigated using additional panels of sera and fresh whole blood samples from TB-free captive (Weybridge) badgers. For 36 sera the DPP VetTB assay (Line 1 only) exhibited a specificity of 100.0 % when read by eye, and 97.2 % (95 % CI: 91.9–100.0) when read using the quantitative reader (applied the working cut-off value obtained from ROC analysis). In 38 fresh whole blood samples from TB-free captive (Weybridge) badgers the DPP VetTB assay exhibited a specificity of 94.7 % (95 % CI: 87.6–100.0), both when read by eye and when read using the quantitative reader (applied the previously established cut-off value). Although the samples were not matched, there was no difference in the specificity of the DPP VetTB assay (Line 1) between fresh and frozen whole blood samples (Fisher’s exact test, P > 0.05) for results read by eye and using RLU values or in the distribution of RLU values (Kolmogorov-Smirnov test, P > 0.05).

3.3. Diagnostic performance of the DPP VetTB assay relative to existing tests in a high bTB prevalence region (Woodchester Park)

Parallel DPP VetTB assay data for serum and whole blood samples, plus contemporaneous diagnostic test results, were available from 251 Woodchester Park badger captures, representing 134 individual animals (median 2 captures per animal; range 1–5). In the case of Line 1 (MPB83) there was a strong association between quantitative results for the DPP assay in serum and whole blood samples (Spearman’s r = 0.57, 95 % CI: 0.48–0.65, P < 0.0001). Notably, there were a significant number of samples for which a non-zero MPB83 result was obtained in whole blood but not serum, indicating the higher average background values generated with this sample type (Fig. 3). The correlation between results in serum and whole blood was not significant in the case of Line 2 (ESAT-6/CFP-10; Spearman’s r = 0.06, 95 % CI: −0.07 to 0.19, P > 0.05).

Cohen’s kappa values for the two methods of interpreting DPP VetTB assay result (read by eye and quantitative reader) indicated almost perfect agreement for Line 1 (0.90 and 0.85 for serum and whole blood respectively). Comparisons between results read by eye and quantitative reader were not performed for Line 2, as its performance did not warrant further investigation. For Line 1, the agreement between sample types (serum and whole blood) was 0.57 (moderate) when read by eye and 0.61 (substantial) when read by quantitative reader. For Line 2, the agreement between sample types (serum and whole blood) was 0.09 (slight) when read by eye.

In this dataset there was no significant difference between the proportion of positive results generated by reading DPP VetTB tests by eye and using the quantitative reader with cut-off values established previously for Line 1 (Fisher’s exact test, P > 0.05 for both serum and whole blood). The level of agreement observed between the VetTB DPP assay (Line 1 only) and contemporaneous diagnostic test data was variable (Table 3). The highest agreement was observed in animals identified as positive on the basis of bacteriological culture of clinical samples, with between 73.7 and 78.9 % of these samples also positive by DPP VetTB assay (Table 3). The largest discrepancy in positive
results was observed between the BrockTB Stat-Pak and DPP VetTB assays, with the former assay identifying a larger proportion of individuals as positive when compared to the DPP VetTB assay in either serum or whole blood (Table 3). In serum from animals that were negative by all contemporaneous test data (culture, interferon-gamma ELISA and BrockTB Stat-Pak) 2.1% and 0.7% were identified as positive by DPP VetTB assay read by eye and quantitative reader, respectively. In whole blood the proportion of otherwise negative samples identified as positive by DPP VetTB assay rose to 9.9% and 4.9% for tests read by eye and by quantitative reader respectively (Table 3).

Cohen’s kappa values calculated for DPP VetTB assay results and contemporaneous data from existing diagnostic tests in badgers from Woodchester Park ranged from fair (0.23) to moderate (0.52). The lowest levels of agreement were observed between DPP VetTB assay and bacteriological culture of clinical samples (kappa between 0.23 and 0.37; see Table 3). However, agreement between bacteriological culture and all formats of the DPP VetTB assay was higher than between culture and the BrockTB Stat-Pak assay (kappa = 0.16). Agreement between the BrockTB Stat-Pak and DPP VetTB assays ranged from 0.44 to 0.48 (moderate).

3.4. Sensitivity and specificity of the DPP VetTB assay in sera from badgers with detailed post mortem results (BVFT)

Quantitative DPP VetTB assay results from 21 TB-free badgers and 30 with culture and/or histologically confirmed M. bovis infection were available for ROC analysis, in order to provide estimates of sensitivity and specificity relative to post mortem data (Fig. 4). In this panel of sera the DPP VetTB assay (Line 1 only) exhibited optimal performance (i.e., maximum likelihood ratio) at a sensitivity of 60.0% (95% CI: 40.6–71.1), and a specificity of 95.2% (95% CI: 85.3–99.6).

In the panel of samples with post mortem confirmation of M. bovis infection (n = 30) the DPP VetTB assay exhibited a sensitivity of 66.7% (95% CI: 49.8–83.5) when read by eye, which was identical to that of the BrockTB Stat-Pak. In the panel of samples from animals confirmed to be free of M. bovis infection (n = 21) the specificity of the DPP VetTB assay was estimated to be 95.2% (95% CI: 86.1–100.0) when read by eye, compared to 100% in the BrockTB Stat-Pak. Agreement between the DPP VetTB (Line 1) and BrockTB Stat-Pak assay...
was substantial (Cohen’s kappa = 0.71 and 0.63 for tests read by eye and quantitative reader, respectively).

4. Discussion

The work presented here provides an initial evaluation of the Chembio Dual-Path Platform (DPP) VetTB assay for the detection of \textit{M. bovis} infection in badgers. We have evaluated the diagnostic performance of the DPP VetTB assay using panels of samples collected from both naturally and experimentally infected badgers, and animals known or suspected to be free of \textit{M. bovis} infection, and compared the assay’s performance with existing diagnostic methods. This study employed a

![Fig. 2](image-url) Performance of the Chembio DPP VetTB assay in a panel of sera (a & b) and whole blood (c & d) from TB-free captive and culture confirmed naturally infected badgers, showing (a & c) log-transformed relative light unit (RLU) values for Line 1 (MPB83) with working cut-off (dashed horizontal line) and mean values (solid horizontal lines) and (b & d) ROC curves based on RLU data.

![Fig. 3](image-url) Antigen specific antibody responses measured by DPP VetTB assay in parallel serum and whole blood samples from 251 badger captures at Woodchester Park. Plotted values show log-transformed relative light units (RLU) for Line 1 (MPB83) and Line 2 (ESAT-6/CFP-10) responses.
Table 3

Agreement between contemporaneous diagnostic test data and DPP VetTB assay results in 251 serum and whole blood samples collected from Woodchester Park badgers. DPP VetTB assay results (Line 1 only) for both serum and whole blood were read by eye and using a quantitative reader to record relative light units (RLU). Agreement is presented as percentage of individuals in each group positive by DPP VetTB assay, relative to contemporaneous diagnostic test results, and measured by Cohen’s kappa statistic.

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Whole blood</th>
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<tr>
<td></td>
<td>Eye</td>
<td>RLU</td>
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<tr>
<td>Culture positive (n = 19)</td>
<td>% pos 73.7</td>
<td>73.7</td>
</tr>
<tr>
<td></td>
<td>Kappa 0.34</td>
<td>0.37</td>
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<tr>
<td>Interferon-gamma ELISA positive (n = 41)</td>
<td>% pos 63.4</td>
<td>63.4</td>
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<td></td>
<td>Kappa 0.49</td>
<td>0.52</td>
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<tr>
<td>BrockTB Stat-Pak positive (n = 101)</td>
<td>% pos 43.6</td>
<td>42.6</td>
</tr>
<tr>
<td></td>
<td>Kappa 0.44</td>
<td>0.45</td>
</tr>
<tr>
<td>Interferon-gamma ELISA and BrockTB Stat-Pak positive (n = 36)</td>
<td>% pos 67.7</td>
<td>69.4</td>
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<tr>
<td></td>
<td>Kappa 0.45</td>
<td>0.44</td>
</tr>
<tr>
<td>Negative by all tests (culture, interferon-gamma ELISA and BrockTB Stat-Pak)</td>
<td>% pos 2.1</td>
<td>2.1</td>
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<td>Kappa –</td>
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a Cohen’s kappa values calculated based on parallel interpretation of interferon-gamma ELISA and BrockTB Stat-Pak assays (see Drewe et al., 2010).

b Not possible to calculate Cohen’s kappa values for animals negative by all tests.

Retrospective design, making use of samples collected during previous and on-going studies, and as such there are a number of limitations. One of these is that it was not possible to entirely standardise the storage of sample panels prior to testing. The duration of serological sample storage may potentially impact sample integrity, with repeated freeze-thawing, in particular, known to adversely impact the performance of antibody-based assays for tuberculosis (Boadella and Gortazar, 2011). In the current study, the impact of freeze-thawing was minimised by aliquoting samples into single-use volumes prior to storage. Additionally, samples were stored under conditions (−80 °C) considered to be optimal for stabilising IgG antibodies (e.g. Neumann and Bonistalli, 2009). Nonetheless, future work should ideally employ a study design which explicitly controls the duration of storage when evaluating the performance of the DPP VetTB assay in different sample panels. Furthermore, in the current study two panels of whole blood samples (Woodchester Park and additional TB-free captive Weybridge badgers) were tested fresh, whereas other whole blood samples were stored frozen prior to testing. Whilst the samples were not matched, there was no evidence of a significant difference in the performance of the DPP Vet TB assay in fresh and frozen whole blood. However, future work should seek to explicitly compare the impact of freeze-thawing whole blood samples on test performance, particularly if the DPP VetTB assay is to be applied as a trap-side test (using freshly collected whole blood samples). A further limitation of the current study is that samples were tested by several different operators, in two separate locations. To mitigate this, data were generated using an established standard operating procedure, with training in the protocol provided to all operators. Additionally, the performance of the optical reader device was calibrated between locations. However, future validation work should seek to explicitly address issues of both intra- and inter-operator reliability.

Initial evaluation of the diagnostic performance of the DPP VetTB assay in badgers was undertaken using samples from experimentally infected animals. This evaluation demonstrated that the antigenic targets included in the two lines of the DPP assay (MPB83 and ESAT-6/CFP-10 fusion protein, respectively) exhibited very different diagnostic performance, with ESAT-6/CFP-10 specific antibody responses showing no significant increase with disease progression. Previous studies have demonstrated that MPB83 specific antibodies are dominant in the serological response to M. bovis infection in badgers (Greenwald et al., 2003; Leselier et al., 2009a, b). However, it has also been shown by multi-antigen print immuno-assay (MAPIA) that a smaller proportion of animals additionally respond to the antigens ESAT-6 and CFP-10, both individually and as a fusion protein (Greenwald et al., 2003; Leselier et al., 2008, 2009a, b). Studies in cattle have demonstrated that individual profiles of reactivity to specific antigens may evolve with disease progression (e.g. Lyashchenko et al., 2017). Data from experimentally infected badgers in the current study show no evidence of a temporal change in responses to the ESAT-6/CFP-10 fusion antigen included in the DPP VetTB assay (e.g. Fig. 1b). However, the relatively short time-scale of the experimental infection protocol used in the current study may not fully replicate the longer-term kinetics of the antibody response exhibited in naturally infected animals. Hence it would be valuable to study the relative performance of sero-diagnostic antigens over a longer period of infection.

Studies in other species have demonstrated that IgM responses to M. bovis infection can be detected earlier than IgG responses, though they also wane more quickly (e.g. Waters et al., 2006; Lyashchenko et al., 2017). For this reason it has been suggested that assays detecting both IgM and IgG responses, such as the Chembio BrockTB Stat-Pak, may exhibit greater sensitivity than those detecting IgG alone, such as the DPP VetTB assay (Waters et al., 2006). In the current study, samples from experimentally infected badgers indicated that detectable MPB83-specific antibody responses in serum developed between two and four weeks after infection in the majority of animals. This is consistent with previous studies of IgG antibodies to MPB83 in experimentally infected

Fig. 4. a) Quantitative DPP VetTB assay Line1 (MPB83) results for BVFT sera with post mortem confirmation of disease status, and (b) ROC analysis based on these data. Values plotted in (a) are log-transformed relative light unit (RLU) values, with working cut-off (dashed horizontal line) and means (solid horizontal lines).
badgers, which demonstrated that responses were first detected by ELISA at three to four weeks following challenge (Lesellier et al., 2008, 2009b). Hence there is no indication, from the current study, that measurement of the IgG antibody responses alone has negatively impacted the ability of the DPP Vet TB assay to detect the early stages of infection.

The current evaluation has allowed us to assess the sensitivity of the DPP VetTB assay relative to the BrockTB Stat-Pak, which is no longer commercially available. It should be noted, however, that the confidence intervals around estimates of sensitivity overlap, and hence we cannot make definitive conclusions on the relative performance of the two assays (DPP VetTB and BrockTB Stat-Pak). This highlights the challenge of generating validation data for diagnostic assays in wildlife populations, where the availability of reference samples from animals of known disease status is often limited (OIE, 2014).

Selecting representative reference samples for the intended validation purpose is an important consideration when evaluating diagnostic assay performance in wildlife species. In particular, samples should be obtained from sub-clinically infected animals if the test undergong validation is to be used in apparently healthy animals (OIE, 2014). In the present study the panel of samples used to generate preliminary estimates of relative sensitivity were collected from naturally infected live badgers, identified by isolation of M. bovis from clinical samples. However, samples from animals in which bacterial excretion has been detected may not be representative of the true range of infection states in a wild population, as these individuals are typically at a more advanced stage of disease progression and hence are also most likely to have a detectable antibody response to M. bovis (e.g. Chambers et al., 2008).

In addition to providing estimates of the sensitivity and specificity of the DPP VetTB assay, we have attempted to assess agreement between this test and existing diagnostic methods used on live animals, with samples from an intensively studied free-living badger population in a high bTB incidence area (Woodchester Park). Agreement, measured as the proportion of animals DPP VetTB assay positive in a group identified by existing test data, was highest for badgers which were culture positive, for all DPP VetTB assay formats (74–79%). The proportion of animals positive by the DPP VetTB assay was lowest in the sub-set of Woodchester Park badgers identified as positive on the basis of contemporaneous BrockTB Stat-Pak results alone (43%–55%, depending on DPP VetTB assay format). Agreement between the two tests was moderate in this group (Cohen’s kappa 0.44–0.48). This result is somewhat unexpected, due to the similarity between the two tests, both of which are lateral-flow format assays detecting antibodies targeting the same sero-dominant antigens. Whilst this may reflect true differences in the relative sensitivity of the assays, other factors may also be of significance. Recent Bayesian analysis of the relative sensitivity of the BrockTB Stat-Pak using data generated in the Woodchester Park population has indicated that the sensitivity of this assay is higher than that demonstrated by previous studies, exceeding that of the interferon-gamma ELISA in this population (Buzdugan et al., 2016). This runs counter to orthodoxy regarding the relative sensitivity of assays based on the humoral and cellular immune responses for detecting TB infection (e.g. Kunnath-Velayudhan and Gennaro, 2011), and hence could conceivably reflect something specific to the application of the BrockTB Stat-Pak assay in the Woodchester Park population. Interestingly, previous studies have demonstrated an increasing temporal trend in sero-prevalence within this population, relative to prevalence based on bacteriological culture (Delahay et al., 2013). Further investigation is required to disentangle the cause, or causes, of the observed discrepancy between BrockTB Stat-Pak and DPP VetTB assay performance in this population.

Some discrepancy was also observed between DPP VetTB assay results and existing assays in badgers from Woodchester Park that were negative by all contemporaneous tests (interferon-gamma ELISA, BrockTB Stat-Pak and bacterial culture). Parallel interpretation of these tests has been estimated to provide a negative predictive value of 97 % (Drew et al., 2010), and hence provides substantial confidence in the probable disease status of this sub-set of animals. Thus the proportion of animals identified as positive by the DPP VetTB assay in whole blood when read by eye (9.9 %), is somewhat higher than predicted by estimates of the assay’s specificity in other panels of samples. One potential explanation for the higher proportion of putative “false-positives” observed in whole blood samples when read by eye is inter-operator differences in the interpretation of the test. Whilst this factor was mitigated as far as possible (see above) it was not explicitly incorporated into the design of the current study, and warrants further investigation.

There is some evidence, from the data presented here, that the specificity of the DPP VetTB assay in sera and whole blood may be higher when using the quantitative reader (applying the working cut-off values established) than when interpreting tests by eye. A number of factors will influence the degree of concordance observed between the two methods. Firstly, the cut-off values applied to quantitative results will clearly influence the number of positive tests identified. Further work, using larger panels of samples and incorporating additional populations, may demonstrate that optimal sensitivity and specificity can be achieved by adjusting the cut-off values applied here. A second set of factors that are likely to significantly influence the degree of concordance observed between DPP VetTB assay results based on quantitative values and when read by eye are the conditions under which the test is performed. If the DPP VetTB assay were applied as a “trap-side” test it is reasonable to assume that it might often be undertaken in poor or inconsistent light conditions. The effect of such variables on the accuracy of reading tests by eye requires further evaluation.

The use of quantitative values for the interpretation of the DPP VetTB assay provides a number of additional benefits. Importantly, quantitative values allow the trade-off between sensitivity and specificity to be adjusted, according to the requirements of the intended application. Where prevalence is relatively high (such as during the initial stages of a disease control programme), it may be desirable to favour higher sensitivity over specificity, in order to identify the maximum number of infected animals. Conversely, specificity becomes more important as prevalence decreases, and in such scenarios it may be desirable to increase specificity at the cost of sensitivity, in order to reduce the number of false positives (Greiner et al., 2000; Buzdugan et al., 2016).

Finally, the current study has confirmed the existence of a small proportion of animals ostensibly free of M. bovis infection which nonetheless produce detectable antibody responses to specific antigens. As described, it is considered unlikely that any of these captive TB-free (Weybridge) animals were harboring an undetected M. bovis infection. Similar false-positive reactions before disease challenge have been seen in previous experimental studies in the UK (Lesellier et al., 2008) and Republic of Ireland (Aznar et al., 2014). The likely explanation for such reactions is serological cross-reacting followed by exposure to related mycobacteria expressing MPB83, or other environmental bacteria expressing a homologous protein. Cross-reactions in serological tests for M. bovis have been reported in a number of species, and attributed to several non-tuberculous mycobacteria (e.g. Buddle et al., 2010). The precise cause of such false-positive reactions in badgers requires further investigation.

5. Conclusions

This study provides an initial evaluation of the performance of the DPP VetTB assay in badgers, which act as a wildlife reservoir of M. bovis. The DPP VetTB assay format possesses a number of characteristics that make it particularly attractive, such as the small sample volume needed, minimal laboratory infra-structure requirements, potential to be applied as a “trap-side” test and the option to test whole blood, as well as serum. Despite the limitations of the study, these results indicate that the DPP VetTB assay provides a useful diagnostic tool, with...
performance characteristics approximately equivalent to those observed in previously described serological assays. However, confidence intervals around estimates of both sensitivity and specificity are currently relatively large, and further work is required to increase confidence in the estimated performance of the DPP VetTB assay. This study highlights the importance of carefully evaluating diagnostic assays for wildlife species in the practical and epidemiological contexts in which they will be applied. This is particularly important if the assay is intended to be used as part of a disease control intervention.

Declarations of Competing Interest

None.

Acknowledgements

This work was funded by the Department for Environment, Food and Rural Affairs, primarily under project SE3281. We are grateful to APHA staff from the Animal Sciences Unit, Pathology Department, National Wildlife Management Centre (Woodchester Park) and Surveillance and Laboratory Services Division who contributed to this work. We are also grateful to Tara Fitzsimons (University College Dublin) for assistance in providing BVFT sera. All animal procedures performed in the UK were covered by licences issued under the Animals (Scientific Procedures) Act, 1986, following local ethical review and approval. Ethical approval for collection of samples in the Republic of Ireland was obtained from the University College Dublin (UCD) Animal Research Ethics Committee (AREC-P-08-26).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.prevetmed.2020.105005.

References


