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Serologic responses correlate with current but not future bacterial shedding in badgers naturally infected with *Mycobacterium bovis*

Elli R. Jolma[1,2] | **Richard J. Delahay**[3] | **Freya Smith**[3] | **Julian A. Drewe**[2]

[1] Institute of Zoology, Zoological Society of London, Regent's Park, London, UK

[2] Veterinary Epidemiology, Economics and Public Health Group, Royal Veterinary College, London, UK

[3] National Wildlife Management Centre, Animal and Plant Health Agency, Woodchester Park, Nympsfield, Gloucestershire, UK

Correspondence
Julian A. Drewe, Veterinary Epidemiology, Economics and Public Health Group, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire, AL9 7TA, UK.
Email: jdrewe@rvc.ac.uk

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Abstract

Bovine tuberculosis is a challenging cattle disease with substantial economic costs in affected countries. Eradication in parts of the United Kingdom and Ireland is hindered by transmission of the causative agent *Mycobacterium bovis* between cattle and European badgers (*Meles meles*). Diagnostic tests in badgers are of limited accuracy but may help us understand and predict disease progression. This study aimed to determine the practical ability of a commercially available serologic test, the Dual Path Platform VetTB assay (DPP), to predict mycobacterial shedding (i.e. infectiousness) and disease progression in badgers, and whether test outcomes were associated with re-capture. Clinical samples collected from 2014 to 2019 from a wild, naturally infected population of badgers in southwest England were tested using mycobacterial culture (from sputum, urine, faeces, abscesses and bite wounds), an interferon-gamma release assay and the DPP assay. Data were analysed at both individual badger and social group levels using generalised linear and cumulative-link mixed models, and linear regression. Only the highest DPP readings [optical density relative light unit (RLU) levels] were associated with mycobacterial shedding [odds ratio (OR) for DPP levels > 100 RLU in individual badgers: 79.6, 95%CI: 14.7–848; and for social groups: OR: 7.28, 95%CI: 2.94–21.44; compared with levels < 100 RLU]. For individual badgers, RLU levels at first capture were not associated with disease progression at subsequent captures. Finally, badgers with very high DPP levels (> 1000 RLU) were four times less likely to be recaptured (OR: 0.24, 95%CI: 0.07–0.83) than those without a detectable DPP response, which might indicate enhanced mortality. We conclude that DPP levels of > 100 RLU identify badgers that are likely to be shedding *M. bovis*. Levels of > 1000 RLU identify badgers that are much less likely to be re-captured. These results provide insights into the potential value of existing tests in intervention strategies for managing *M. bovis* in badgers.

KEYWORDS
bacterial shedding, badgers, epidemiology, *Mycobacterium bovis*, serologic tests

1 | INTRODUCTION

Pathogen transmission between wildlife, livestock and humans can have significant impacts on agriculture, animal and public health, and conservation (Daszak et al., 2000). Bovine tuberculosis (bTB) is caused by the zoonotic pathogen *Mycobacterium bovis* and is an important infection of cattle in many parts of the world. In the United Kingdom the disease is at the root of an expensive and controversial human–wildlife conflict (Cassidy, 2012) because infection can be maintained and transmitted in both directions between European badgers (*Meles meles*) and cattle (Crispell et al., 2019). Effective control of bTB in cattle is therefore likely to require some interventions to reduce transmission risks from badgers. Diagnostic tests may play an important role in the management of disease in badger populations, either for surveillance purposes or to identify infected animals for selective interventions.

There are three types of diagnostic test available for bTB in live badgers, but all have their limitations. Culture of clinical samples (i.e. sputum, faeces, urine, wound swabs) has the highest specificity (just under 100%), but the lowest sensitivity (only 8%–10%) (Drewe et al., 2010), and is not suitable for selective management interventions because badgers shed bacteria only intermittently and obtaining culture results requires weeks (Chambers et al., 2009; Clifton-Hadley et al., 1993). The gamma-interferon release assay (IFNγ) that measures cell-mediated immunity (Dalley et al., 2008) is moderately specific (around 95%) and more sensitive (80%) than culture (Drewe et al., 2010). IFNγ tests are useful at predicting future disease progression (Buzdugan et al., 2017a; Tomlinson et al., 2015), but require laboratory processing and are therefore unsuitable for trap-side testing (Chambers, 2009; Tomlinson et al., 2015). Serologic tests are less specific than culture and less sensitive than IFNγ assays but can produce rapid results in the field (Chambers, 2009; Chambers et al., 2009). A previously available serologic test (Stat-Pak) showed improved sensitivity in badgers with progressed disease (approx. 88%) compared with those at an earlier stage of infection (approx. 58%), with approximately 93% specificity in all adult badgers regardless of disease stage (Chambers et al., 2009). Due to the limited sensitivity of all these tests at the individual level, their utility has been considered in combination with other tests (Drewe et al., 2010) and to estimate infection status at the level of the badger social group (Buzdugan et al., 2016).

The Dual Path Platform VetTB test (DPP) has recently replaced the Stat-Pak serologic test (Ashford et al., 2020; Courcier et al., 2020). The DPP is a lateral-flow test with antigens MPB83 and CFP10/ESAT-6 bound to two test lines (Waters et al., 2017). Antibody binding to these antigens can be detected by eye or with an optical reader that produces a quantitative optical density measured in relative light units (RLU) (Waters et al., 2017). In badgers, only the test line containing MPB83 antigen (line 1) has been shown to have diagnostic value, with a suggested cut-off of 71.7 RLU for classifying badgers as infected using serum samples giving a sensitivity of 55.3% and specificity of 98.1% (Ashford et al., 2020). Whole blood samples provide comparable results with a higher cut-off of 154 RLU with a sensitivity of 52.5% and specificity of 98.1% (Ashford et al., 2020). The DPP test clearly has potential value but there are some unanswered questions relating to its practical utility in badgers. We sought to address some of these questions in the present study. Specifically, we asked whether DPP test results could be used to: (1) identify the current bacterial shedding status (i.e. infectiousness) of an individual badger; (2) predict future disease progression of a badger; (3) estimate bacterial shedding at the level of the badger social group rather than the individual; and (4) determine whether DPP RLU levels are related to the probability of a badger being recaptured. The latter (4) is based on a previous finding by Buzdugan et al. (2017b) that infected badgers were less likely to be recaptured than uninfected animals. Knowledge of this is required to determine if our disease progression model (2) is reliable and if trapped animals provide a representative sample of the whole population. The ability to estimate individual- and group-level bacterial shedding based on DPP levels would be valuable for disease surveillance in badgers and could potentially be used to inform targeted interventions.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The authors confirm that the study complies with the ethical policies of the journal and that the capture and sampling of badgers was approved by the Animal Welfare Ethical Review Board of the Animal and Plant Health Agency and conducted in accordance with Natural England and UK Home Office licences.

2.2 | Data collection

Data came from the long-term study of a wild badger population at Woodchester Park, Gloucestershire, UK (see McDonald et al., 2018). Four times per year, baited cage traps were set near the active setts (underground burrow systems) in the territories of each badger social group in the study area. Captured animals were anesthetised, and clinical samples collected for *M. bovis* culture, IFNγ and serologic tests (see Drewe et al., 2010 for details). Sex and age group (cub = under 1 year, adult = 1 year and above) were recorded for each animal. The present study used data from animals sampled from 20 May 2014 to 11 September 2019. Only captures for which results were obtained for all three diagnostic tests (culture, IFNγ and serology) were included.

Microbiological culture was performed on sputum collected by tracheal and oesophageal aspiration, urine, faeces, pus from abscesses and swabs from bite wounds. The IFNγ test was performed on fresh heparinised whole blood, which was incubated with bovine and avian tuberculin in separate tubes resulting in IFNγ production following the protocol of Dalley et al. (2008). The optical density (OD) of the reaction to the bovine tuberculin minus the reaction to the avian tuberculin was measured following the same protocol. We used a cut-off of 0.044 OD for both adults and cubs to classify an animal as infected, which gives a sensitivity of 80.9% and specificity of 93.6% according to Dalley et al. (2008). Both serum and whole blood can be used for the DPP test (sample preparation and test method described in detail by Ashford et al.,

TABLE 1 Definitions for the four infection status categories of individual badgers used in this study

Infection status category	Culture result	IFNγ OD level	Number of observations (captures) in this study	Corresponding categorisation from Delahay et al. (2000)
0	All culture results negative	<0.044	859	Not exposed
1	All culture results negative	≥0.044	275	Exposed
2	One positive culture result	Any value	45	Excretor
3	Two or more positive culture results (either at the same or different capture events)	Any value	24	Super-excretor

IFNγ = interferon gamma release assay.
Note: A badger could only progress forwards on the infection status scale and so could not return to any lower category. The IFNγ value is the optical density (OD) of the reaction to purified protein derivative of bovine tuberculin minus the reaction to purified protein derivative of avian tuberculin.

2020) but for the present study we used serum. The majority (56%, $n = 675$) of our serum samples were frozen before analysis. Only results from DPP test line 1 (i.e. MPB83 antigen) were used, based on the findings and recommendations of Ashford et al. (2020) and Courcier et al. (2020).

2.3 | Data analysis

To explore the relationship between the results of the DPP and the two other diagnostic tests, we derived a new variable called 'infection status', based on IFNγ and culture results (Table 1). This proxy for true infection status was adapted from the 'disease status' categorisation used in Delahay et al. (2000) and is based on the assumption that a badger can only progress forwards in terms of infection status, meaning that it could not return to any lower category. To determine if DPP RLU categories could predict disease progression over time, a new variable called 'disease progression' was created by subtracting the infection status (Table 1) at a badger's first capture during the study from its infection status at each subsequent capture. Thus, a value for 'disease progression' was generated for each badger every time it was captured after its first capture in the dataset. All positive values were interpreted as indicating disease progression between the first and the subsequent capture.

Because some serum samples were frozen and defrosted before testing, we checked for differences in DPP RLU distributions between fresh and frozen samples by plotting histograms for each infection status and running a Kolmogorov–Smirnov test (Dodge, 2008). Serologic results (DPP RLU levels) were allocated to categories (Table 2) because they had a zero-inflated distribution with positive values approximating a log-normal distribution (1st quartile: 0, median: 2.5, 3rd quartile: 23, maximum value: 6925). This categorisation was based on the total distribution of values and the distribution of values across each of the four infection status categories (Table 1). Table 2 shows how positive *M. bovis* culture results were distributed across the DPP categories.

We used three types of model to answer four research questions (see below and Table 3). The suite of models provided a comprehensive understanding of how each DPP RLU level related to infection status and culture positivity, of individual badgers and of animals in the same social group. Some of our models used DPP cut-offs (i.e. thresholds; for example badgers with RLU levels at or above 71.7 RLU; as per Ashford et al., 2020) and some used DPP categories (ranges; for example badgers with RLU levels from 100 to 1000 RLU). All analyses were conducted in R version 3.5.3. (R Core Team, 2019). The package *ordinal* version 2019.12-10 was used for all cumulative link mixed models (Christensen, 2019), *lme4* version 1.1–23 for all generalised linear mixed models (GLMM) with Laplace approximation (Bates et al., 2015) and *ggplot2* for data visualisation (Wickham, 2016).

TABLE 2 DPP test categories used for the individual-level analyses

DPP category	Level (RLU)	Number of observations (captures)	Number (%) of captures with a positive *M. bovis* culture result
0	0	522	14 (3%)
1	>0–20	360	7 (2%)
2	>20–50	100	5 (5%)
3	>50–100	56	1 (2%)
4	>100–1000	82	8 (10%)
5	>1000	83	34 (41%)

DPP = Dual Path Platform VetTB assay; RLU = relative light unit reading of the optical density of the DPP line 1 (MPB83 antigen).

2.4 | Question 1. Do DPP RLU levels correlate with current mycobacterial infection or shedding status of an individual badger?

To allow graphical exploration of trends, all RLU levels were increased by 0.05 (to account for zero-inflation) and log-transformed. A histogram with Wilcoxon rank sum test was drawn to explore crude associations between continuous RLU levels and infection status categories. Subsequently, the association between RLU categories and infection status was tested with a chi-square test.

Temporal variation in RLU levels for individual badgers was explored using a GLMM (Model 1 in Table 3). RLU levels that reduce to zero in

TABLE 3 Overview of the structure and composition of statistical models and their relationship with the four research questions addressed in this study

Model number	Research question addressed	Focus of analysis	Model type	Response investigated	Fixed effects	Random effects
1	Q1. Do DPP RLU levels correlate with the current mycobacterial infection or shedding status of an individual badger?	Temporal variation of DPP RLU levels (RLU levels that reduce to zero in subsequent captures may suggest these badgers were never infected, based on the assumption that infected badgers never clear *M. bovis* infection).	GLMM	Non-zero DPP RLU level in the subsequent capture	DPP RLU category in the first capture of the study period	Individual badger
2		Associations between DPP RLU levels and infection status	Cumulative-link mixed model	Infection status category	DPP RLU category, age, (sex)	Individual badger (nested in the social group)
3		Associations between DPP RLU levels and concurrent mycobacterial shedding	GLMM	Positive culture result	DPP RLU category, age, (sex)	Individual badger (nested in the social group)
4	Q2. Do DPP RLU levels predict disease progression in individual badgers?	Associations between DPP RLU levels and disease progression over time	GLMM	Disease progression within 6, 9, 12, 18 and 24 months after the first capture	DPP RLU category in the first capture of the dataset,age	Individual badger
5	Q3. Do DPP RLU levels correlate with mycobacterial shedding at the badger social group level?	Associations between DPP RLU levels in individual badgers and concurrent group-level mycobacterial shedding	GLMM	At least one culture-positive badger in the group that year	At least one badger over one of the cut-offs that year. Cut-offs: DPP RLU > 0, 71.7, 100, 1000 and visible visual line 1	Social group
6		Associations between DPP RLU levels in individual badgers (with cut-offs) and concurrent mycobacterial shedding	GLMM	Positive culture result	DPP RLU cut-offs > 0, 71.7, 100, 1000 and visible visual line 1, age	Individual badger
7	Q4. Is the probability of a badger being recaptured related to DPP RLU levels or the results of cultures or the IFNγ test?	Associations between test results in the first capture of the study period and the badger being captured again	Logistic regression	The badger being re-captured	Results from the first capture during the study: Positive culture, RLU category, IFN$\gamma \geq 0.044$. Model run separately for cubs and adults.	Not applicable

Note: Variables in parentheses were dropped from the final models. Infection status categories are described in Table 1, and DPP categories in Table 2. GLMM = generalised linear mixed model; DPP = Dual Path Platform VetTB serologic assay; RLU = relative light unit reading of the optical density of the DPP line 1 (MPB83 antigen); IFNγ = interferon gamma release assay value (optical density of the reaction to purified protein derivative of bovine tuberculin minus the reaction to purified protein derivative of avian tuberculin)

subsequent captures may suggest these badgers were never infected, based on the assumption that infected badgers never clear *M. bovis* infection. Variables that did not have significant parameter estimates ($p > .1$) were dropped when the simpler model had a lower Akaike information criterion (AIC) value. Our model selection criteria was based on a review by Johnson and Omland (2004) .

At the individual level, associations between the DPP and other diagnostic test results within sampling events (i.e. at the same time point) were explored using two types of model. A cumulative link mixed model fitted with flexible threshold (CLMM) was used to detect associations between RLU categories and badger infection status (Model 2 in Table 3). A GLMM was used to establish associations between RLU categories and bacterial shedding (Model 3 in Table 3). Age was initially tested both as an individual fixed effect and as an interaction term for RLU categories. Variables that did not have significant parameter estimates ($p > .1$) were dropped when the simpler model had a lower AIC value.

2.5 | Question 2. Do DPP RLU levels predict disease progression in individual badgers?

The association between DPP RLU levels in the first capture of the dataset and disease progression in individual badgers during subsequent captures was investigated using GLMMs (Model 4 in Table 3). Models were conducted for captures within 6, 9, 12, 18 and 24 months after the first capture. The models for longer follow-up times included the captures of the shorter follow-up times because of the small number of observations in each time period. The small number of observations also led to only variables that were significant ($p < .1$ and lower AIC value in the simpler model) in Models 2 and 3 being included as random and fixed effects in the disease progression models (details in Table 3).

2.6 | Question 3. Do DPP RLU levels correlate with mycobacterial shedding at the badger social group level?

Social group-level analyses were conducted using five separate GLMMs: one for each of four machine-read RLU cut-offs for the DPP test (> 0, 71.7, 100 and 1000 RLU) and one for the visual detection of the test line (Model 5 in Table 3). The response variable for all these models was the presence of at least one culture-positive badger in the social group during that year. The RLU categories in Table 2 were not used for the group-level analyses due to the limited number of observations (143 observations of 26 social groups; each group having observations from 2 to 6 years). Only the last capture of each badger in each year was included in the analysis to avoid repeated measures. To investigate how the number of badgers with DPP test results above the RLU cut-off related to presence of a positive culture result in the group, we also ran GLMMs that were otherwise identical to Model 5, but included the number of badgers over the cut-off as the response.

In order to allow direct comparison of DPP RLU levels with culture positivity at both the individual and group level, we performed five individual-level GLMMs with the same five RLU cut-offs as above (Model 6 in Table 3). This was because the main individual-level analysis used categories instead of cut-offs.

2.7 | Question 4. Is the probability of a badger being recaptured related to DPP RLU levels or the results of cultures or the IFNγ test?

Associations between DPP RLU categories at a badger's first capture and whether it was recaptured during the study period, were investigated using logistic regression (Model 7 in Table 3). Associations were also investigated between positive culture results and recapture, and between IFN$\gamma \geq 0.044$ OD and recapture. In these analyses, only those animals first captured in the study period before 1 January 2018 ($n = 251$) were included to allow for a follow-up time of at least 18 months. Age group at first capture had significant interaction ($p = .02$ in the likelihood ratio test of models with and without interaction) with infection status in the recapture analysis. Therefore, recapture results were calculated for cubs and adults separately instead of using age group as an independent variable.

3 | RESULTS

3.1 | Overview

The dataset for analysis consisted of 1203 captures of 353 badgers from 26 social groups. The median number of captures per badger was 2, ranging from 1 to 16 (mode = 1). The mean number of different badgers caught in each social group was 17.2, ranging from 6 to 31 (this distribution approximated normal: Shapiro–Wilk test $p = .52$). Of all capture events, 68% ($n = 814$) involved adult badgers, and 53% ($n = 187$) of all individuals were female. DPP results from fresh and frozen serum had similar distributions of RLU levels (Kolmogorov–Smirnov test, $p = .22$) as suggested by visual observation of histograms of levels stratified by infection status category (Figure S1). Consequently, DPP RLU results from fresh and frozen sera were pooled for analysis.

Badger social group was not a significant random effect in any individual-level model (Models 2 and 3: see Table 3); using only the individual badger resulted in lower AIC values than the models using both individual and social group as random effects. Therefore, all final individual-level models had only the individual as a random effect and all group-level models had only the social group as a random effect. Sex was not a significant fixed effect in any model ($p > .1$). Age group was a significant fixed effect in most individual-level models ($p < .1$), but it had a significant interaction only in the recapture model (Model 7 in Table 3) for infection status categories (likelihood-ratio test for the model with interaction term, and using age as a separate explanatory variable: χ^2 with 2 df = 7.76, $p = .02$); consequently, all recapture models were conducted for cubs and adults separately.

TABLE 4 Summary interpretation of DPP test RLU levels, and their associations with other diagnostic tests for tuberculosis in badgers, based on the findings of this study

DPP category	DPP level (RLU)	Interpretation
1	>0–20	No associations with other tests. Zero levels often seen in subsequent captures.
2	>20–50	Weak positive association with infection status category, but not with culture as a standalone variable.
3	>50–100	Weak positive association with infection status category, but not with culture as a standalone variable.
4	>100–1000	Weak positive association with a positive culture result as a standalone variable. No effect on recapture.
5	>1000	Strong positive association with a positive culture result. Negative association with subsequent recapture of the same badger.

DPP = Dual Path Platform VetTB assay; RLU = relative light unit reading of the optical density of the DPP test line 1 (MPB83 antigen).

3.2 | Summary interpretation of DPP serologic test levels

An overview of this study findings is given in Table 4. This summarises the DPP results and their associations with other diagnostic tests for tuberculosis in badgers. Results are explained in more detail below, under each of the four research questions.

3.3 | 1. Do DPP RLU levels correlate with current mycobacterial infection or shedding status of an individual badger?

In individual badgers, only the two highest DPP RLU categories were associated with positive *M. bovis* culture results (Table 5): category 4 (DPP > 100–1000 RLU) had a borderline significant association [odds ratio (OR): 24.5, 95% confidence intervals (CI): 1.91–671] and category 5 (DPP >1000 RLU) had a clear association (OR: 9070, 95%CI: 364–3.12e+08), albeit with very wide confidence intervals (due to the low number of positive culture results). All categories of DPP RLU levels above 20 and the 'adult' age group were positively associated with infection status (Table 5).

DPP RLU levels differed significantly between badgers in infection status categories 0 and 1, and between categories 1 and 2 (Wilcoxon rank sum test $p < .001$), whereas the difference between categories 2 and 3 was non-significant ($p = .681$) (Figure S2). Nevertheless, only category 3 had a non-zero first quartile (31.71 RLU) indicating that DPP test results of 0 RLU were rare in badgers in the highest infection status category, but were common in all lower infection status categories (Figure S2). DPP RLU categories were significantly associated with infection status ($\chi^2 = 319.31$, df = 15, $p < .001$) and culture negative captures had a lower median DPP RLU value (1st quartile: 0, median: 2, 3rd quartile: 192, maximum value: 6618) than culture-positive captures (1st quartile: 6, median: 970, 3rd quartile: 4754, maximum value: 6925).

Both the graphical exploration (Figure S3) and the model for DPP RLU temporal variations (Table S1) showed that RLU levels change over time in individual badgers. In particular, animals with levels below 20 RLU had a tendency to produce zero levels when tested at subsequent captures: the confidence interval for RLU category 1 overlapped with that of the intercept (Table S1). Age at capture was not a significant fixed effect for RLU change over time (parameter estimate $p = .77$, AIC for the simpler model 1.9 units smaller).

3.4 | 2. Do DPP RLU levels predict disease progression in individual badgers?

DPP RLU levels at the first capture event during the study period were not significantly associated with subsequent disease progression (GLMM model 4: all $p > .05$) as measured by subtracting the infection status (Table 1) at the first capture from infection status at each subsequent capture. This was the case for captures taking place within 6, 9, 12, 18 and 24 months after the first capture of the same badger (not all badgers were caught at all these time intervals, so sample size varied for each of these analyses).

3.5 | 3. Do DPP RLU levels correlate with mycobacterial shedding at the badger social group level?

Badger group-level analyses of bacterial shedding produced similar results to individual-level analyses, but with narrower confidence intervals (Table 6). At the group level, associations were detected between at least one badger having a positive culture result in any given year and at least one badger having a positive DPP result using cut-offs of 71.7 (OR 7.47, 95% CI 2.88–23.71), 100 (OR 7.28, 95% CI 2.94–21.44) and 1000 RLU (OR 11.04, 95% CI 4.26–36.57). There was also an association between bacterial shedding and DPP test line 1 being visible to the naked eye (OR 7.78, 95% CI 2.82–27.76) (Table 6). There was a consistent trend for the presence of higher numbers of badgers over each DPP cut-off or with a visible DPP test line to result in a stronger association with group-level culture positivity, although confidence intervals overlapped (Table S2).

The results of individual-level models could be directly compared with those from group-level models where the same DPP cut-offs were used (Table S3 compiles the calculated odds from Tables 6 and S2). Such comparisons indicate that when a badger has a DPP RLU level of >1000, the odds for at least one other badger in that social group having a positive *M. bovis* culture result that year are relatively high (odds = 1.41). In contrast, the odds for the badger itself having a positive culture result are substantially lower (odds = 7.58e-04). The

TABLE 5 Individual-level associations between serologic test result (DPP test result categories) and (a) positive Mycobacterium bovis culture result and (b) *Mycobacterium bovis* infection status category in badgers

	Odds ratio (95% confidence interval) for positive culture	Model estimate (*p*-value) for infection status
DPP category 1 (>0–20 RLU)	0.9 (0.09–8.2)	0.16 ($p = .58$)
DPP category 2 (>20–50 RLU)	6.9 (0.8–91.3)	0.92 ($p = .04$)
DPP category 3 (>50–100 RLU)	60.8 (0.7–49500)	1.32 ($p = .03$)
DPP category 4 (>100–1000 RLU)	24.5 (1.9–671)*	2.55 ($p = 1.70e-07$)
DPP category 5 (>1000 RLU)	9070 (364–3.12e+8)***	5.29 ($p = 2e-16$)
Age group adult	95.4 (5.4–74000)*	2.84 ($p = 8.68e-14$)
Intercept	2.10e-08 (6.95e-14–3.94e-6)	Not applicable

Note: These results were generated using models 2 and 3 (see Table 3 for details of models). The intercept is DPP test result category zero and the reference age group is cub. Infection status categories are based on a combination of results from culture and interferon gamma release assay (described in Table 1), and DPP categories are described in Table 2.
DPP = Dual Path Platform VetTB assay; RLU = relative light unit reading of the optical density of the DPP test line 1 (MPB83 antigen).
Significance levels: *$p < .05$, **$p < .01$, ***$p < .001$.

TABLE 6 Associations between DPP test result (using a range of RLU cut-offs) and positive *Mycobacterium bovis* culture in social groups and individual badgers

Badger social group level					
	DPP serologic test result				
	Test line visible to the naked eye	**Relative light unit cut-off level**			
		>0 RLU	**>71.7 RLU**	**>100 RLU**	**>1000 RLU**
Odds ratio	7.78	3.80	7.47	7.28	11.04
95% confidence interval	2.82–27.8	0.68–71.6	2.88–23.7	2.94–21.4	4.26–36.6
Significance level	$p < .001$	N.S.	$p < .001$	$p < .001$	$p < .001$
Odds	0.64	–	0.66	0.73	1.41
Individual badger level					
	DPP serologic test result				
	Test line visible to the naked eye	**Relative light unit cut-off level**			
		>0 RLU	**>71.7 RLU**	**>100 RLU**	**>1000 RLU**
Odds ratio	179	12.6	74.4	79.6	3070
95% confidence interval	21.4–4840	2.8–82.6	13.3–877	14.7–848	150–1.4e+07
Significance level	$p < .001$	$p < .01$	$p < .001$	$p < .001$	$p < .001$
Odds	1.07e-05	5.922e-07	1.07e-05	2.05e-05	7.58e-04

Note: Group-level results are odds ratios for at least one badger in the same social group developing a positive culture result in the same year; these results were generated with Model 5 (see Table 3 for details of models). Individual-level results were generated with Model 6. Note that the odds ratios originate from separate models each with its own intercept; therefore, the odds for each association (calculated from the odds ratio and the model intercept) are also presented to allow comparison between models. The intercept of each social group model was zero badgers in the group having a DPP RLU level over the cut-off and the intercept of each individual-level model was a badger with RLU level below the cut-off.
DPP = Dual Path Platform VetTB assay; RLU = relative light unit reading of the optical density of the DPP test line 1 (MPB83 antigen).

confidence intervals were overlapping between group- and individual-level analyses due to the very wide confidence intervals of the individual-level results. The main difference between individual-level analyses with RLU categories (Table 5) and RLU cut-offs (Table 6) was that using cut-offs made any positive RLU level appear to have an association with a positive culture result, whereas DPP category analysis detected an association only with the two highest RLU categories (i.e. >100 RLU).

3.6 | 4. Is the probability of a badger being recaptured related to DPP RLU levels or the results of cultures or the IFNγ test?

Adult badgers with the highest DPP RLU levels (category 5: >1000 RLU) were over four times less likely to be recaptured than were badgers with the lowest DPP levels (category 0) (OR 0.24, 95% CI 0.07–0.83) (Table 7). Adult badgers with a positive culture result were over

TABLE 7 Associations between the results of three different diagnostic tests for *Mycobacterium bovis* infection in badgers at the time of first capture, and the badger being subsequently re-captured during this study

	Odds ratio (95% confidence interval) for recapture of same badger	
	Cubs	Adults
IFNγ optical density ≥0.044	0.15 (0.04–0.56)**	0.54 (0.24–1.2)
Positive culture of *M. bovis*	2600000 (0–∞)	0.18 (0.04–0.91)*
DPP category 5 (>1000 RLU)	6650000 (0–∞)	0.24 (0.07–0.83)*

Note: These results were generated using Model 7 (see Table 3 for details of models). The reference value for each explanatory variable was a negative test result (for the DPP test this was category 0). DPP test RLU result categories are described in Table 1.
DPP = Dual Path Platform VetTB assay (a serologic test); RLU = relative light unit reading of the optical density of the DPP line 1 (MPB83 antigen); IFNγ = interferon gamma release assay value (optical density of the reaction to purified protein derivative of bovine tuberculin minus the reaction to purified protein derivative of avian tuberculin).
Significance levels: *$p < .05$, **$p < .01$, ***$p < .001$.

five times less likely to be recaptured than were badgers with a negative culture result (OR 0.18, 95% CI 0.04–0.91) (Table 7). Cubs with a positive IFNγ test result were over six times less likely to be recaptured than were cubs with a negative IFNγ test result (OR 0.15, 95% CI 0.04–0.56) (Table 7).

4 | DISCUSSION

The present study found a relationship between high antibody levels (DPP > 100 RLU) and shedding of *M. bovis* (i.e. infectiousness) in naturally infected badgers. This association builds on previous findings, which showed that the sensitivity of Stat-Pak, another serologic test, was highest in badgers in advanced stages of *M. bovis* infection (Chambers et al., 2008). Our results suggest that interpreting DPP results from serum based on absolute RLU levels instead of using a single cut-off of 71.7 RLU (as recommended by Ashford et al., 2020) could be beneficial in situations where higher test specificity and information about likely shedding are required (Schiller et al., 2010), whereas the recommended cut-off (71.7 RLU) appears to have use in detecting infected badgers. A requirement for higher test specificity could arise for example, towards the end of an eradication campaign where prevalence and positive predictive value are both low. In a recent study, badgers that tested positive with the DPP test (based on visual interpretation) were culled but only 48% of these animals were culture-positive at necropsy (Courcier et al., 2020). This suggests that some badgers with positive DPP results may have been false positives, given that the study used a highly sensitive (although not perfect) necropsy culture protocol similar to that of Murphy et al. (2010). This is not a criticism of the study by Courcier et al. (2020) because we acknowledge they used the best available cut-off at that time, with no prior knowledge of prevalence. Our findings suggest that in a similar situation, the use of an optical reader with a cut-off of 100 RLU could result in fewer false positives, although this might be accompanied by an increase in false negatives (lower sensitivity).

Comparing individual-level DPP results reveals how cut-off usage can be potentially misleading. The individual-level models using *RLU cut-offs* (Table 6) detected significant associations with culture for all positive DPP RLU cut-offs; whereas models using *DPP categories* (Table 5) found associations with culture only for the two highest categories [and the lowest category (category 1) did not differ from zero levels in any test]. This effect of the choice of whether to use cut-offs or categories is important to understand whenever using the DPP to obtain a binary outcome; especially if an optical reader is not available in the field and decisions are based on visual interpretation of the test line (e.g. Courcier et al., 2020). According to Ashford et al. (2020), in serum samples the visual interpretation of test line 1 shows good agreement with optical reader results using a cut-off value of 71.7 RLU. The categorisation of RLU values in the present study was problematic as the small number of observations between 100 and 1000 RLU did not allow for more categories in that range. Nonetheless, it is important to appreciate that 100 is not a definitive cut-off over which all levels are strongly associated with positive cultures, but rather a starting point above which results begin to become relevant to the goal of detecting badgers that may be shedding mycobacteria. As the association with culture was quite strong for DPP RLU levels over 1000, it would be beneficial to explore in more detail the relationship within the range 100–1000 RLU.

Positive culture results from clinical samples indicate that a badger is actively shedding mycobacteria into the environment, but such results were rare in our dataset, and this is likely partly due to the low sensitivity of culture (Drewe et al., 2010) and partly to the intermittent shedding of mycobacteria (Clifton-Hadley et al., 1993). This means that it is more reliable to measure mycobacterial shedding at the badger group level rather than at the individual level (Buzdugan et al., 2016), because badgers live in territorial social groups and the presence of an infected individual markedly increases the likelihood of infection for other group members (Vicente et al., 2007). This is reflected in the present study, where there was a stronger association between DPP results and culture results at the group, rather than the individual level. The odds for having at least one culture-positive animal in the group when at least one member of that group had a DPP result >1000 RLU were 1.41, whereas the odds for the same badger having a positive culture result were only 7.58e-04. This group-level interpretation helped to mitigate the very wide confidence intervals associated with individual-level analyses of culture results. The main limitation of our group-level model was the small number of observations that limited our analyses to a binary cut-off interpretation and that resulted in the loss of range-specific information. Our results also provide an interpretation for situations when DPP test results are available for several badgers from the same social group.

Our simple model did not take the size of the social group into account and it is possible that the proportion (rather than absolute

number) of badgers in the group that yield a DPP result over the cut-off point may be a better predictor of infectiousness at the group level. However, using a pre-specified threshold value is likely to be more practical in the field because group size is usually unknown. Group-level DPP interpretation could be useful as a surveillance tool to relatively rapidly determine if a local badger group is likely to be shedding *M. bovis*, which might be used to inform targeted intervention measures (vaccination or culling for example). Limitations of this approach include the challenge of capturing sufficient badgers, and the risk of creating counter-productive perturbation effects which have been associated with increased bTB incidence in cattle in adjacent areas (Donnelly et al., 2006).

In the present study, DPP results did not predict disease progression in individual badgers. This was expected in the case of IFNγ results because cell-mediated responses typically precede serologic responses (Maas et al., 2013); although, in experimentally infected badgers both cellular response and antibody production for MPB83 occurred at the same time about six weeks post-infection (Lesellier et al., 2008). In contrast, the potential for DPP test results to predict positive culture results was deemed more likely because of the possible early antibody response for MPB83 (Lesellier et al., 2008). However, the absence of any such predictive association in the present study may relate to the observation of MPB83 responses being most pronounced in badgers with advanced bTB (Chambers et al., 2009), suggesting that high DPP RLU levels would likely occur concurrently rather than prior to a positive culture result. Our ability to detect any association between DPP antibody levels and future disease progression could also have been limited by the low number of positive cultures and the lower likelihood of recapture amongst culture-positive animals.

The lower recapture probability of adult badgers with positive culture results and high DPP RLU levels (i.e. >1000) is consistent with a previous investigation of the same study population, which found that infected badgers were four times less likely to be re-captured than uninfected badgers (Buzdugan et al., 2017b). In the earlier study, infection was determined probabilistically using a combination of results from three diagnostic tests and several ecological variables. The authors hypothesised that infection-induced changes in badger behaviour may have been responsible for this phenomenon, with clear implications for disease control interventions. In a separate study, badgers that were shedding *M. bovis* were observed to exhibit different ranging behaviour to culture negative animals (Garnett et al., 2005). However, in contrast to the analyses performed by Buzdugan et al. (2017b), in the present study it was not possible to rule out the possibility that reduced re-capture probability arose because high RLU levels were also associated with enhanced mortality, which has been shown to occur for badgers with multiple culture-positive sites (Graham et al., 2013).

There are some important limitations in the approach taken in the present study. Ashford et al. (2020) showed that DPP test results are comparable between different sample types, but recommended a higher cut-off for whole blood than for serum. Therefore, while the trends in our DPP findings from serum samples are likely to apply for whole blood, the exact ranges should be interpreted with caution. Furthermore, the infection status variable used here was likely to categorise some infected animals as uninfected, because the IFNγ response can wane with infection progression, and culture has low sensitivity (Drewe et al., 2010; Maas et al., 2013). Including serologic results (as in Delahay et al., 2000) may have increased confidence in this variable, but would have hampered the independence of the response from our variable of interest (the serologic test result).

5 | CONCLUSIONS

Our study suggests that in naturally infected wild badgers, only the highest DPP test RLU levels (>100) appear to be a reasonable proxy for badgers that are detected as shedding (i.e. infectious, and therefore at risk of transmitting *M. bovis* to others), but that DPP test results are not predictive of future mycobacterial shedding. It is possible that some badgers with DPP RLU levels <100 may still be infectious (but missed by our analysis) because mycobacterial culture of clinical samples is very insensitive. Badgers testing culture-positive in our study were likely to have been shedding relatively large amounts of bacteria (sufficient to be detected) whereas other animals with lower levels of shedding (but still infectious) may have evaded detection. Badgers with very high RLU levels (>1000) not only had a strong likelihood of shedding but also a much-reduced likelihood of being recaptured. This suggests that such animals may be lost to follow-up, perhaps because of enhanced mortality or infection-induced behavioural changes. These findings could therefore have implications for culling strategies that rely on trapping, and research studies which aim to better understand the epidemiology of infection in badgers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Elli R. Jolma https://orcid.org/0000-0002-9356-2891
Julian A. Drewe https://orcid.org/0000-0003-0709-8066

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