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The impact of BCG strains and repeat vaccinations on immunodiagnostic tests in Eurasian badgers (*Meles meles*)

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ABSTRACT

Bacille Calmette-Guerin (BCG) is a potential tool in the control of *Mycobacterium bovis* in European badgers (*Meles meles)*. A five year Test and Vaccinate or Remove (TVR) research intervention project commenced in 2014 using two BCG strains (BCG Copenhagen 1331 (Years 1–3/ BadgerBCG) and BCG Sofia SL2222 (Years 4–5). Badgers were recaptured around 9 weeks after the Year 5 vaccination and then again a year later.

The Dual-Path Platform (DPP) Vet TB assay was used to detect serological evidence of *M. bovis* infection. Of the 48 badgers, 47 had increased Line 1 readings (MPB83 antigen) between the Year 5 vaccination and subsequent recapture. The number of BCG Sofia vaccinations influenced whether a badger tested positive to the recapture DPP VetTB assay Line 1 ($p < 0.001$) while the number of BadgerBCG vaccinations did not significantly affect recapture Line 1 results ($p = 0.59$). Line 1 relative light units (RLU) were more pronounced in tests run with sera than whole blood. The results from an in_house MPB83 ELISA results indicated that the WB DPP VetTB assay may not detect lower MPB83 IgG levels as well as the serum DPP VetTB assay. Changes in interferon gamma assay (IFN-γ) results were seen in 2019 with significantly increased CFP-10 and PPDB readings.

Unlike BadgerBCG, BCG Sofia induces an immune response to MPB83 (the immune dominant antigen in *M. bovis* badger infection) that then affects the use of immunodiagnostic tests. The use of the DPP VetTB assay in recaptured BCG Sofia vaccinated badgers within the same trapping season is precluded and caution should be used in badgers vaccinated with BCG Sofia in previous years. The results suggest that the DPP VetTB assay can be used with confidence in badgers vaccinated with BadgerBCG as a single or repeated doses.

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1. Introduction

The European badger [*Meles meles*] is implicated as a wildlife host and reservoir of *Mycobacterium bovis* infection for cattle in the British Isles. Bacille Calmette-Guerin (BCG) in badgers has been shown to induce protective immune responses to *M. bovis* and is increasingly used as a control measure (as reviewed by Buddle et al. and Robinson et al. [40;7]).

BCG has been used successfully in several wildlife species other than badgers such as brushtail possums [*Trichosurus vulpecula*] [32], white tailed deer [*Odocoileus virginianus*] [34], and wild boar [*Sus scrofa*] [3]. The recent Test and Vaccinate or Remove (TVR) research project in Northern Ireland demonstrated a significant fall in *M. bovis* prevalence in the badger population over a five year period [1,29].

There are a number of strains of BCG vaccine. All BCG strains derive from the original BCG vaccine strain produced at Pasteur Institute of Lille, France in the early part of the twentieth century [4]. The strain was then distributed worldwide and propagated on several non-synthetic culture media with different passaging schedules leading to a number of genetically distinct daughter strains. In the 1960s, lyophilisation was introduced to store these seed lots preventing further BCG sub-strain diversity.

Despite more than fourteen current sub-strains in existence, only five major sub-strains are used in current vaccine production;

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Brazilian (Moreau/Rio de Janeiro), Danish (Copenhagen – 1331), Japanese (Tokyo – 172-1), Russian (Moscow – 368) and Bulgarian (Sofia – SL222) (see [4]). These can be divided into two groups; those derived in the early 1920s (including BCG Russia) (i.e. early strains) and those derived from strains originating at the Pasteur Institute after 1927 (including BCG Copenhagen) (i.e. late strains). Late strains appear to be associated with decreased production of antigens MPB70, MPB83, and MPB64 [4].

BCG Sofia is in the same lineage as BCG Moscow and is regarded as functionally indistinct [42,35]. Approximately 30 countries use BCG Moscow/Sofia as their sole BCG strain for medical vaccinations [38] and it is one of the three strains supplied by Unicef. BCG Copenhagen was derived from a strain (passage 423) received by Statens Serum Institut (Denmark) in 1931. In 1960, lyophilisation was carried out after passage 1331 and this formed the primary seed lot in 1966 [33]. BCG Copenhagen 1331 strain is the only commercially available BCG vaccine licensed for medical procedures in the European Union and is used in 32 countries [38]. It is also the strain in the sole UK licensed parenteral BCG vaccine for badgers (BadgerBCG; a live attenuated lyophilised vaccine with 2–8 $\times$ 10^6 cfu BCG Copenhagen 1331) [6]. There have been several worldwide shortages of BCG vaccine supply since 2008 (for further discussion see [9]. These resulted in supply issues with BadgerBCG and BCG Sofia (SL222- BullBio-NCIPD) was sourced as a replacement BCG vaccine for the last two years of the TVR project (2017 and 2018).

The Dual-Path Platform (DPP) VetTB assay was used to detect *M. bovis* infection in badgers during the TVR project [29]. It is a single use, point of care, immune-chromatographic (lateral-flow) rapid assay for the detection of antibodies to *M. tuberculosis* and *M. bovis* in cervid serum. Two recombinant antigens (MPB83 and CFP10/ESAT-6 fusion) proteins are immobilised on the test strip as separate lines (Line 1 and Line 2, respectively). MPB83, a 25 kDa protein, is sero dominant in infected badgers [14,17,24]. Recent studies have given confidence in the use of the DPP VetTB assay in free living badgers [2,10,1,23]. Parallel interpretation of Line 1 and 2 was found not to be diagnostically better than use of Line 1 only [10]. Previous analysis had also indicated that changing from BadgerBCG to BCG Sofia had a significant impact on DPP VetTB serum assay sensitivity and specificity [1]. There is little other literature surrounding the effect of BCG vaccination on diagnostic tests for *M. bovis* in badgers. In this paper, our objective was to investigate the effect of BCG strain type and revaccination on sero-diagnostic tests in 48 badgers from the TVR project. The results will inform future vaccination and testing strategies for *M. bovis* control in badgers and help in the design of badger prevalence studies after vaccination campaigns.

2. Materials and methods

A TVR wildlife research intervention project was performed under licence in a 100 km^2 area in County Down, Northern Ireland (for full description see [29]). The intervention employed the Dual Path Platform (DPP) VetTB assay for cervids (Chembio Diagnostic Systems Inc. New York, USA) as the field diagnostic tool. All badgers were individually identified by microchip on first capture. During the first year (2014), all captured badgers were sampled and tested, vaccinated with BadgerBCG and released. In the following four years, the TVR approach was employed where DPP VetTB assay positive badgers were removed and DPP VetTB assay negative badgers were vaccinated and released. Due to supply issues with BadgerBCG, BCG Sofia was used in 2017 and 2018 (Year 4 and 5). This research operated under the Animals (Scientific Procedures) Act 1986 (as amended) - 'ASPA'. The ASPA licences were issued to Department of Agriculture, Environment and Rural Affairs (DAERA) by the Department of Health, Social Services and Public Safety (DHSSPS) in Northern Ireland (Project Licence Numbers 2767 and 2872). Licences were also obtained from Northern Ireland Environment Agency (NIEA) to allow the capture, sampling, collaring and removal of badgers.

2.1. Additional recapturing

To evaluate the effect of BCG Sofia, a sub-area of the TVR study area was retrapped in October 2018 (7–10 weeks after the initial TVR round of trapping). This sub-area was also retrapped one year later (September 2019). No badgers were removed during these two retrapping periods but badgers that were identified as having received at least one vaccination with BCG Sofia were blood sampled.

2.2. Diagnostic tests

Following capture and identification, badgers were anaesthetised using an intramuscular injection with a triple combination of ketamine hydrochloride (Narketan, Vetoquinol UK Ltd), medetomidine hydrochloride (Domitor, Vetoquinol UK Ltd) and butorphanol tartrate (Torbugesic, Zoetis UK Ltd). The first time a badger was captured, a microchip was inserted subcutaneously between the shoulder blades to enable future identification. Blood was taken via the jugular vein into heparinised vacutainer tubes and in Serum Separation Tubes for diagnostic testing. A tracheal aspirate and an oropharyngeal swab for culture were collected from each badger up to Summer 2018. Swabs were also taken for culture from any bite wounds observed on the animal. The blood samples from all animals underwent field whole blood (WB) DPP VetTB assay testing and gamma-interferon (IFN-γ) testing, WB DPP VetTB and serum DPP VetTB assays under laboratory conditions. In the field, the WB DPP VetTB assay was carried in an insulated heated box and on a level surface to ensure optimal conditions with visual readings from Line 1 or Line 2 recorded separately. An optical reader in the laboratory was employed to obtain quantitative readings for the WB DPP VetTB and serum DPP VetTB assays. The IFN-γ assay was conducted using heparinised whole blood, following the protocol described in [11], using purified protein derivative of M. *bovis* (PPDB) and purified protein derivative of M. *avium* (PPDA) and CFP-10 (Prionics Lelystad B.V., Lelystad, Netherlands). Supply issues with the DPP VetTB assay kits occurred in 2017 and 2018 and a proportion of whole blood samples were not tested with the DPP VetTB assay in the laboratory in those years.

In addition to DPP VetTB assay testing, the anti-MPB83 serostatus of serum samples taken in 2019 were also characterised on an optimised anti-MPB83 ELISA previously established in-house for use in another mustelid species, ferrets (*Mustela furo*) – see Corbett *et al. Unpublished results*. ELISA plates were coated with 100 ng/well of an in-house produced MPB83 antigen diluted in carbonate-bicarbonate buffer (Sigma-Aldrich C3041) and incubated for 1 h at 37 °C. After the plates were washed, 100 µL serum from each test sample (diluted 1/10, 1/100 and 1/1,000 in PTN buffer consisting of PBS (Sigma-Aldrich 806552), Tween20 (AppliChem A7564) and NaCl (Sigma-Aldrich S7653)) was added in duplicate to the plate along with a negative control and positive control. Plates were incubated for 1 h at 37 °C and then washed. Bound antibodies were identified using goat anti-ferret IgG labelled with HRP (abcam ab112770, diluted 1/40,000 in PTN buffer) following incubation of 1 h at 37 °C and then washed. Finally, the substrate TMB/E (Millipore ES001) was added and the reaction stopped with 0.5 M H_2SO_4. The absorbance of each sample was read at 450 nm using a Tecan Sunrise plate reader. Samples were designated seropositive where the difference in optical density (OD) between 1/10 and 1/1000 dilutions was > 0.2. In addition, rel-

ative quantitative ELISA Unit (EU) values were calculated using a calibrated standard curve established using pooled seropositive serum.

2.3. Data analysis

The effects of vaccination (number of doses and different strain types) were explored on the results of the serum and WB DPP VetTB assays, IFN-γ assay and MPB83 assay. The data were manipulated in Microsoft Office Access 2013 and analysed in R version 3.5.0 [37]. Plots were generated using *ggplot2* package [45]. Mann Whitney tests were used for comparing group median values. For analyses of previous results between 2017 and 2019, badgers were grouped according to the number of years vaccinated and the BCG strain(s) received. One badger was removed from these analyses due to it being the only individual to have received two BadgerBCGs and one BCG Sofia vaccine. The medians and 95% confidence intervals were plotted over time for each group. The medians and confidence intervals were calculated using bootstrapping with 10,000 replicates (as recommended by McGuinness et al. [30]). Correlations between continuous variables were assessed via Pearson's correlation coefficient.

Statistical modelling was used to test whether continuous values for the DPP VetTB assay, IFN assay and MPB83 assay varied over the period BCG Sofia was used (the period between 2017 and 2019). Nonlinear mixed models were built using *nlme* package [36] and the model output was produced using the sjPlot package [27]. Continuous variables were log10 + 1 transformed. Badger identity number acted as the random effect to compensate for the repeat testing of individuals. Potential explanatory effects added to the model were vaccination (years vaccinated, number of BadgerBCG vaccinations received, number of BCG Sofia vaccinations received) and years sampled (2017, 2018, 2018 resampling and 2019). Badgers were only vaccinated in 2018 at the July to September capture. A dummy composite variable (Number of BCG Sofia vaccinations/Year) was also created to avoid singularity within the mixed models. Variables that were significant at $p < 0.05$ or that contributed to a significant improvement in the fit (assessed via AIC) were retained in the model. Diagnostic residual plots were used to assess the fit of the final models.

3. Results

The dataset consisted of 48 animals (23 females and 25 males) that were captured twice during 2018 (once between July and September (first capture 2018) and then again in October 2018 (second capture 2018)) - Fig. 1. At first capture of 2018 (July to September) when the BCG Sofia vaccination took place, all badgers had been swab/aspirate, field DPP VetTB assay and IFN-γ assay negative. Fifteen of these animals were classified as cubs. There was a median of 49 days between the BCG Sofia vaccination in 2018 and the recapture in October 2018 (Interquartile range (IQR) 1 day) and a median of 330 days (IQR 2 days) between the retesting in October 2018 and September 2019.

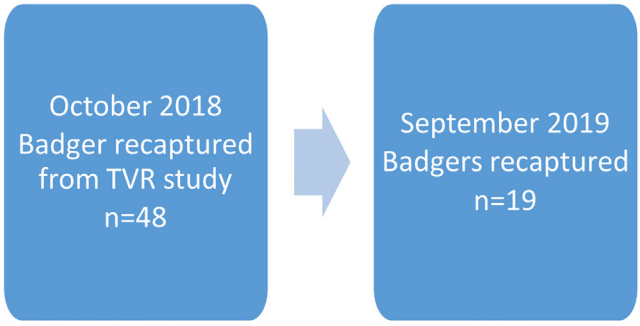

Fig. 1. Data used in study.

3.1. Effect of BCG Sofia on WB DPP VetTB assay results

When animals were captured for a second time in 2018, the majority were Line 1 positive in the field (63% -30/48). None were Line 2 positive. The lab WB DPP VetTB assay results confirmed these findings. Line 1 relative light unit (RLU) values were significantly higher at the second capture in 2018 compared to all previous years and to 2019 ($p < 0.001$, Median second capture = 6.08 (IQR 4.6), 2014–2018 median = 0 (IQR = 0), 2019 median = 0 (IQR = 1) –Fig. 2A. By 2019, only 4 animals were field positive at Line 1 (4/19) and two were Line 2 positive.

BCG Sofia induced a high Line 1 RLU within 3 months of vaccination, but a year later the effect had subsided (Fig. 2A). This was not seen for Line 2 (Fig. 2B). Badgers were grouped according to BCG strain (Sofia or BadgerBCG) and number of BCG vaccinations received by 2019 (BCG Sofia was used in 2017 and 2018, BadgerBCG was used in 2014 to 2016) creating four groups. At the second capture in 2018, those badgers that were vaccinated twice with BCG Sofia had higher Line 1 RLUs than those only vaccinated once (Mann Whitney Test $p < 0.001$) – Fig. 2A. This RLU rise was estimated to be around 1500% for those badgers vaccinated twice compared to those vaccinated once by the log-linear model (Table 1). By 2019, the model suggested around a 85% reduction in RLU values for these double vaccinated badgers when compared to single vaccinated badgers in 2018. There was no statistically significant difference in Line 1 RLU values in 2019 between badgers that had previously received BadgerBCG and those that had not ($p = 0.57$). No differences were evident between groups for WB Line 2 RLU values in either October 2018 or 2019 ($p = 0.64$, $p = 0.57$ -Fig. 2B).

3.2. Effect of BCG Sofia on serum DPP VetTB assay results.

Ten percent (5/48) of badgers tested positive on serum DPP VetTB assay Line 1 when were first caught in 2018. Forty nine days later, three quarters of badgers tested positive on Line 1 (77%, 37/48) and nearly all badgers had increases in serum DPP VetTB assay Line 1 RLU values (47/48) – Fig. 2C. 33 of these 37 visually positive badgers (89%) had previously tested serum DPP VetTB negative at first capture in 2018. No animals were serum DPP VetTB assay Line 2 positive at first capture in 2018 while one animal was tested serum DPP VetTB assay Line 1 and Line 2 positive at second capture in 2018. Eight animals (42%) were serum DPP VetTB assay positive when tested in 2019.

There was no difference in 2018 first capture Line 1 RLUs or Line 2 RLUs between badgers that had been vaccinated with BCG Sofia in 2017 and those that had not (2018 Line 1 RLU Vaccinated in 2017 Median = 0 IQR = 11.8, Not vaccinated in 2017 Median = 0 IQR = 0, $p = 0.07$; 2018 Line 2 RLU Vaccinated in 2017 = Median = 0 IQR = 0.51, Not Vaccinated in 2017 = Median = 0 IQR 1.96)- Fig. 2C and D. The proportion of animals assessed as Line 1 positive at first capture in 2018 was not significantly different according to whether they had been vaccinated in 2017 (No 6% n = 1/17, Yes 13% n = 4/31, $p = 0.79$). The number of BadgerBCG vaccinations previously received did not affect Line 1 RLUs ($p = 0.59$) or Line 2 RLUs at second capture in 2018 ($p = 0.54$) (Table 2) or in 2019 (Line 1 RLU $p = 0.19$, Line 2 RLU $p = 0.2$).

Badgers vaccinated twice with BCG Sofia appeared to have higher Line 1 RLUs than those vaccinated once. 97% (30/31) of badgers vaccinated in 2017 and 2018 with BCG Sofia were serum DPP VetTB assay Line 1 positive at the second capture in 2018

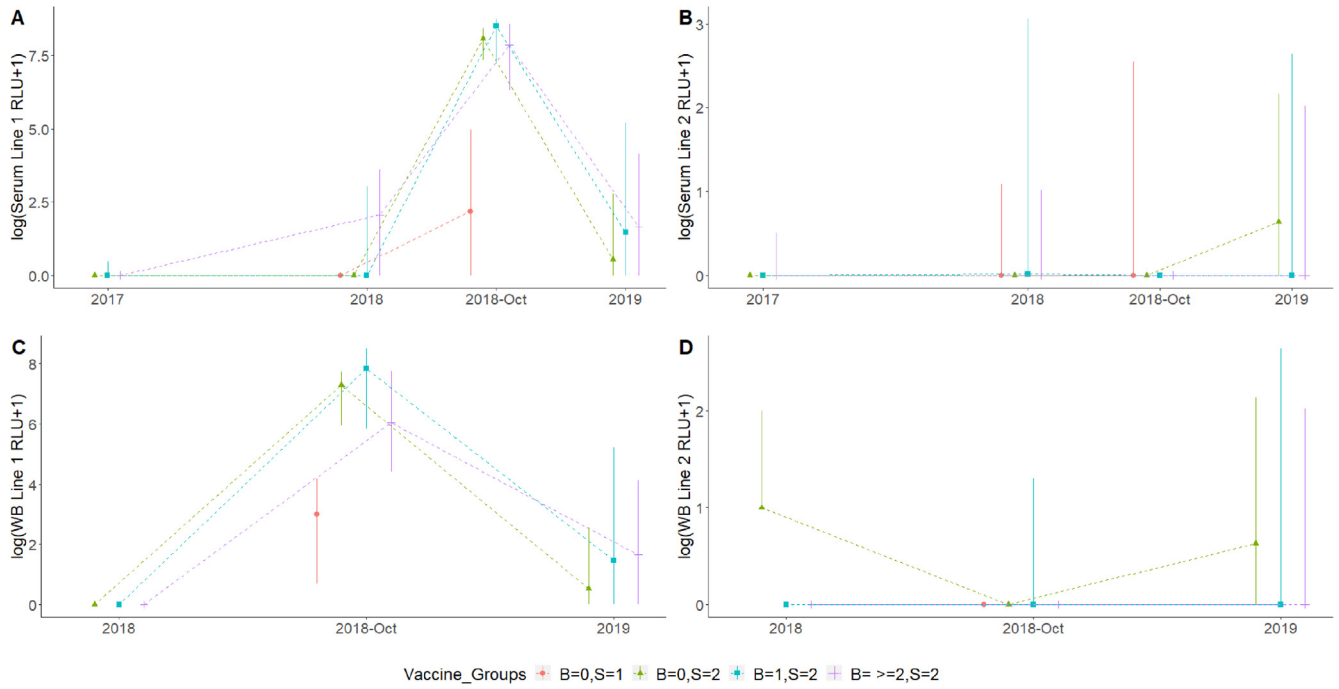

Fig. 2. Plots of bootstrapped whole blood DPP VetTB assay RLU readings. **A and B:** Plot of bootstrapped WB DPP VetTB assay Line 1 and Line 2 RLU readings. **C and D:** Plot of bootstrapped Serum DPP VetTB assay Line 1 and Line 2 RLU readings. Groups correspond to number of BadgerBCG vaccines and number of BCG Sofia vaccinations by 2019 e.g B = number of BadgerBCG vaccine, S = number of BCG Sofia vaccines (B = 0/S = 1n = 16, B = 0/S = 2n = 13,.B = 1/S = 2n = 8 and B >=2/S = 2n = 10). Date range restricted to 2018 onwards for whole blood samples due to missing data. (RLU = relative light units).

Table 1
Results of log(DPP VetTB assay WB Line 1 + 1) mixed model for whole blood DPP VetTB assay Line 1 RLU readings. Data restricted to second capture in 2018 and 2019 due to missing data in 2017 and first capture in 2018. (RLU = relative light units).

	Log(WB Line 1 RLU + 1)		
Predictors	*Estimates*	*CI*	*P*
(Intercept)	3.28	1.99 – 4.56	<0.001
Number of Sofia vaccinations/Year of capture			
1 dose /2018 1st capture	REF		
2 doses /2018 2nd capture	2.79	1.13 – 4.44	**0.002**
2 doses /2019	−1.80	−3.58 –-0.01	**0.049**
Random Effects			
σ^2	3.31		
$\tau_{00\ ID2}$	3.21		
ICC	0.49		
$N_{\ ID2}$	47		
Observations	66		
Marginal R^2 / Conditional R^2	0.548/ –		

Table 2
Results of log(DPP VetTB assay Line 1 + 1) mixed model for serum DPP VetTB assay Line 1 RLU readings. (RLU = relative light units).

	Log(Line 1 RLU + 1)		
Predictors	*Estimates*	*CI*	*P*
(Intercept)	0.32	−0.37 – 1.01	0.367
Number of Sofia vaccinations/Year of capture			
No doses/2017	REF		
No doses/2018 1st capture	0.04	−1.14 – 1.22	0.946
1 dose/2018 1st capture	0.98	0.13 – 1.82	**0.023**
1 dose /2018 2nd capture	2.96	1.78 – 4.14	**<0.001**
2 doses/2018 2nd capture	7.13	6.29 – 7.97	**<0.001**
2 doses /2019	1.23	0.25 – 2.22	**0.014**
Random Effects			
σ^2	2.87		
$\tau_{00\ ID2}$	0.97		
ICC	0.25		
$N_{\ ID2}$	47		
Observations	144		
Marginal R^2 / Conditional R^2	0.657 / 0.744		

compared to 41% (7/17 of badgers vaccinated only in 2018 (Chi square test p < 0.001). Those badgers vaccinated with BCG Sofia in 2017 and 2018 also had statistically significant higher RLUs than those vaccinated in 2018 only (Vaccination 2018 only median RLU = 20.20 (IQR = 142.5), Vaccination 2017 and 2018 RLU = 3178.2 (IQR = 3557.8), Mann Whitney test p < 0.001). All animals captured in 2019 had received two BCG Sofia vaccinations so comparisons were not possible.

At first capture in 2018, badgers vaccinated in 2017 with BCG Sofia did not differ from those not vaccinated with BCG Sofia (Mann Whitney test p = 0.98) – Fig. 2C and D. At the second capture in 2018, those badgers that were vaccinated twice with BCG Sofia had higher Line 1 RLUs than those only vaccinated once (Mann Whitney Test p < 0.001) – Fig. 2C. There was no statistically significant difference in Line 1 RLU values in either capture in 2018 or in 2019 between badgers that had previously received BadgerBCG and those that had not (p = 0.22;p = 0.21). Also no differences were evident between groups for serum Line 2 RLU values (Fig. 2D).

The final loglinear mode (Table 2) demonstrated that the serum DPP VetTB assay Line 1 RLU reading increased in those animals receiving BCG Sofia. Previously BCG Sofia badgers that were captured in summer 2018 had over 165% RLU statistically significant increase when compared to those badgers captured in 2017 with no history of BCG Sofia. At the second capture 2018, badgers that had received two BCG Sofia vaccinations had significantly higher Line 1 RLUs than those that had received one BCG Sofia vaccination. Line 1 RLU readings were also still over 250% higher in 2019 compared to 2017.

3.3. Effect of BCG vaccination on IFN-γ assay results

After no significant changes in IFN-γ CFP-10 and IFN-γ PPDB results in 2017 and 2018 for any of the groups, there was an upward trend between 2018 and 2019 for CFP-10 and PPDB measurements (Fig. 3A and B). No differences between years were apparent for IFN-γ PPDB-PPDA for any of the vaccination groups (Fig. 3C).

Significant small increases for both IFN-γ CFP-10 and IFN-γ PPDB measurements in 2019 were seen in the mixed models (Table 3). BCG Sofia vaccination was not found to be statistically significant. Also no variables were found to be significant for the PPD B-PPDA model.

3.4. Results of anti-MPB83 ELISA in 2019

The majority of badgers tested positive to MPB83 assay in 2019 (13/19, 68%). Of these thirteen, 5 badgers were field and serum DPP VetTB assay positive while 3 were serum positive only. One animal was field DPP VetTB assay positive but MPB83 negative. Strong correlations were seen between MPB83 assay standardized optical density (OD) readings and Serum and WB DPP VetTB assay Line 1 RLUs (Serum rho = 0.91 (95% CI 0.78–0.97); WB rho = 0.79 (95 % CI 0.53–0.92)). WB DPP VetTB assay Line 1 measurements were at lower RLUs than serum values for the same MPB83 OD readings – Fig. 4.

4. Discussion

Vaccination with BCG Sofia invokes a large serological response within three months to MPB83 (the immune dominant antigen in *M. bovis* infection in badgers [24]). This results in animals testing DPP VetTB assay Line 1 positive and was seen with both serum and WB samples. The number of BCG Sofia vaccinations increased the serological response to this antigen within the same trapping season. A smaller rise in Line 1 was also seen one year later in

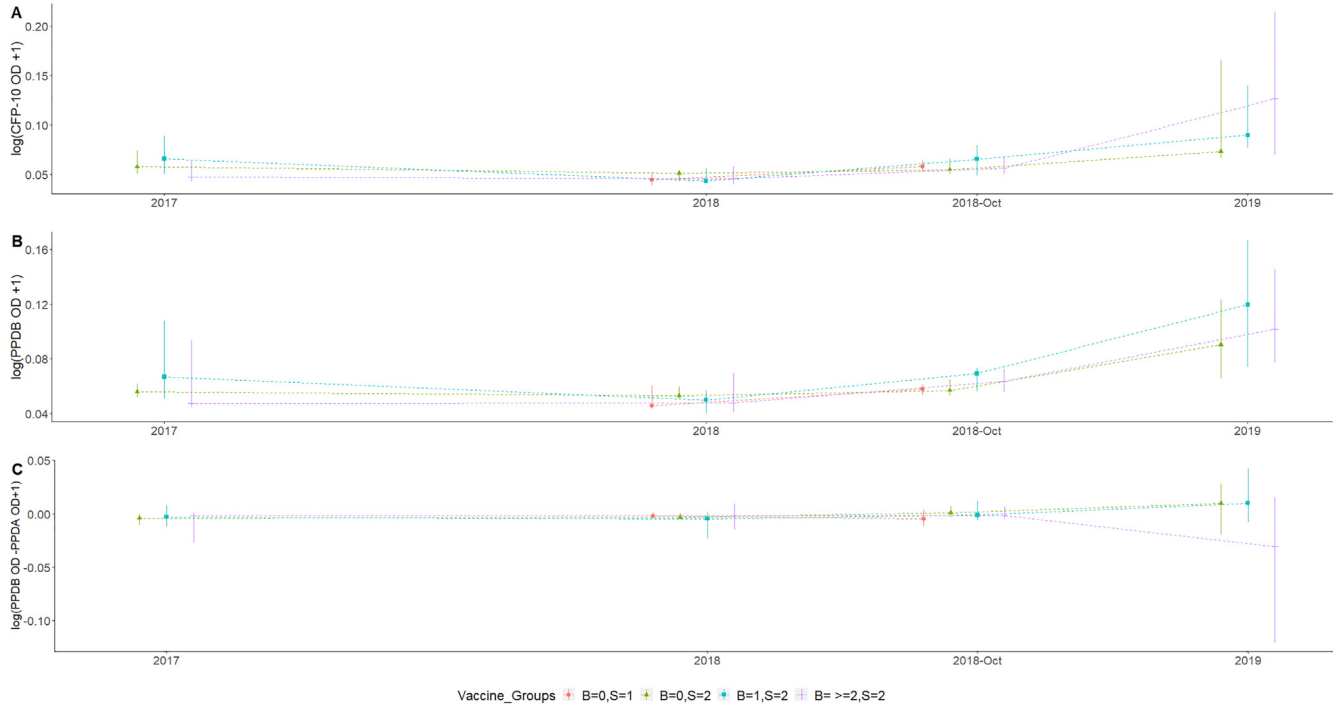

Fig. 3. Plots of bootstrapped IFN-γ measurements. Groups correspond to number of BadgerBCG and number of BCG Sofia vaccinations by 2019 e.g B = number of BadgerBCG vaccines, S = number of BCG Sofia vaccines (B = 0/S = 1n = 16, B = 0/S = 2n = 13,.B = 1/S = 2n = 8 and B >=2/S = 2n = 10). **A:** Plot of boot strapped log(CFP-10 OD + 1) readings. **B:** Plot of boot strapped log(PPDB OD + 1) readings. **C:** Plot of boot strapped log((PPDB OD -PPDA OD) + 1) readings.

Table 3
Results of log(CFP-10 OD + 1) and log(PPDB OD + 1) mixed models.

	Log(CFP-10 OD + 1)			Log(PPDB + 1)		
Predictors	*Estimates*	*CI*	*P*	*Estimates*	*CI*	*p*
(Intercept)	0.03	0.02 – 0.03	**<0.001**	0.03	0.03 – 0.03	**<0.001**
Year [2017]	REF			REF		
Year [2018]	−0.00	−0.01 – 0.00	0.161	−0.01	−0.01 – -0.00	**0.006**
Year [2018 resampling]	0.00	−0.01 – 0.01	0.890	−0.00	−0.01 – 0.00	0.129
Year [2019]	0.03	0.02 – 0.03	**<0.001**	0.02	0.01 – 0.02	**<0.001**
Random Effects						
σ^2	0.00			0.00		
τ_{00}	0.00			0.00		
ICC	0.03			0.01		
N	48			48		
Observations	145			145		
Marginal R^2 / Conditional R^2	0.317 / 0.340			0.323 / 0.333		

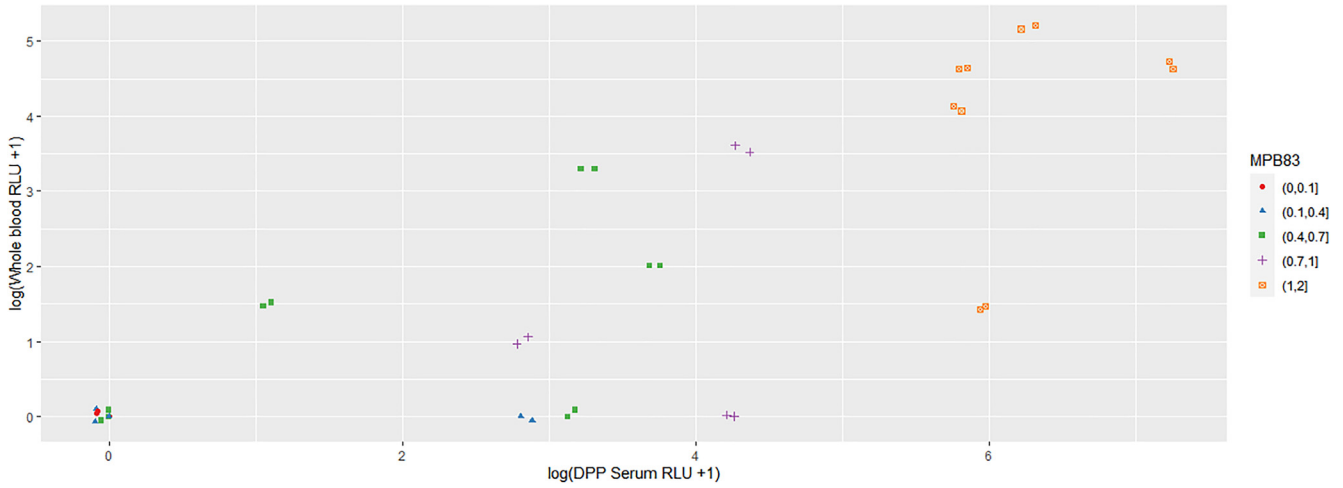

Fig. 4. Scatter plot of log(DPP Serum VetTB assay Line 1 RLU + 1) against log(WB DPP VetTB assay Line 1 RLU + 1). Points categorised by log(MPB83 + 1) optical density (OD reading). (RLU = relative light units).

2019 for badgers that had received two BCG Sofia vaccinations. No such effect was seen for Line 2 of the DPP VetTB assay (ESAT6-CFP10 fusion protein).

This serological response to BCG Sofia was to be expected. Previous studies have demonstrated differential expression of MPB83 in BCG strains [22,46] with a clear delineation between strains obtained from the Pasteur Institute up to 1927 (high-producers) and strains originating in 1931 or later (low-producers) [46]. Badgers vaccinated with BadgerBCG were not tested within one trapping season. However previous studies support the finding of no effect of BadgerBCG on the performance of the DPP VetTB assay. Southey et al. [41] found that the antibody recognition of MPB83 was not significantly enhanced by vaccination with BCG Pasteur which is closely related to BadgerBCG. Gormley et al. [15] used seroconversion detected by BrockTB Stat-Pak lateral flow device (Chembio Diagnostic Systems, New York, USA) as evidence of *M. bovis* infection in badgers vaccinated orally with BCG Copenhagen strain 1331. BrockTB Stat-Pak, the prerunner to the DPP VetTB assay, was used to test badger serum for IgM and IgG antibodies to the antigens MPB83, ESAT-6 and CFP10. They found that vaccinated badgers had significantly lower rates of and a longer time to seroconversion than non vaccinated badgers. Lesellier et al. [26] also demonstrated that the majority of BCG Pasteur 1174P (a low producer like BCG Copenhagen) vaccinated badgers were also not seropositive for rMPB83 on ELISA.

These results also provide the biological basis to some of the findings of Arnold et al. [1] who described how BCG Sofia vaccination lead to a higher sensitivity and a lower specificity for DPP VetTB serum assay but not with the WB DPP VetTB assay in TVR. However this analysis did not include data from the later captures (2nd capture in 2018 and 2019). We found that Line 1 RLUs from both WB and serum samples were raised following BCG Sofia vaccination but this was more pronounced in tests run with sera. The MPB83 assay results indicated that the WB DPP VetTB assay may not detect lower MPB83 IgG levels as well as the serum DPP VetTB assay. This may lead to a greater reduction in sensitivity but an increase in specificity for BCG Sofia vaccinated animals tested with serum DPP VetTB assay than with DPP WB VetTB assay. Further research is needed to explore these differences and their practical implications.

A low serological response was seen to Line 2 (ESAT-6/CFP10 antigens) that was unaffected by repeated vaccinations of BadgerBCG and/or BCG Sofia. This is to be expected as disruption of the ESAT-6 / CFP 10 region (RD1 sequence) occurred early in the development of BCG [21]. However a small significant response to CFP-10 was seen in the IFN-γ assay in 2019. The IFN-γ test based on ESAT6 and CFP10 is recognised to be less sensitive and more specific that the IFN-γ (PPDB – PPDA) test and is used as a DIVA test in cattle [43]. BCG vaccination in badgers is associated with reduced seroconversion (MPB83) and lack of disease progression [8].

Repeated vaccination with BCG Sofia induced a greater serological response to Line 1 (MPB83) in the same trapping season. This complicates the use of the DPP VetTB assay in individuals in the months following the first BCG Sofia dose and in animals that have received multiple BCG Sofia vaccinations. Those animals that had been vaccinated twice rather than once with of BCG Sofia produced a larger response to Line 1 at second capture in 2018. This suggests that immunological memory was improved by this booster in this population. Evidence in humans has shown that a booster dose does not improve efficacy [13]. However, Griffin et al. [18] found that two vaccinations of BCG Pasteur 1173P2 administered with an 8 week interval to red deer [*Cervus elaphus*] were superior to one dose of BCG in reducing subsequent infection.

There were small increases in IFN-γ responses to CFP-10 and PPDB in the years following vaccination with BCG Sofia. No detectable effect in IFN-γ results following BCG vaccination was found by Murphy et al. [31] following oral vaccination with BCG Pasteur and BCG Copenhagen. Lesellier et al. [26] found no responses to BCG vaccination in IFN-γ assays to PPDB with BCG Pasteur 1174P. However, badgers vaccinated had earlier responses to IFN-γ -PPDB than the non vaccinated group to challenge suggesting that immune priming had occurred. There is evidence from human trials that early BCG strains such as BCG Sofia produce a stronger cytokine response than later strains [12,39,28].

The results of this study need to be interpreted with caution. The badgers within this study were free-living and therefore exposed to *M. bovis* and environmental mycobacteria species. Environmental mycobacteria such as *M. kansasii* can elicit reactions to MPB83 and confound serological tests [44]. However, we would have expected that infection pressure for environmental mycobacteria would have not differed significantly throughout the study period and therefore this is unlikely to have affected our results. Our findings are also supported by experimental work carried out in ferrets (Corbett *et al. Unpublished results*).

5. Conclusions

Unlike BadgerBCG, BCG Sofia induces an immune response to MPB83 (the immune dominant antigen in *M. bovis* badger infec-

tion). These findings demonstrate the differential impact of BCG strain and repeat vaccination on the DPP Vet-TB assay and IFN-γ assays in badgers. The use of the DPP VetTB assay to monitor *M. bovis* infection in recaptured BCG Sofia vaccinated badgers within a three month period may be contraindicated. Caution is also needed in badgers that received BCG Sofia in previous years especially when using serum samples.

Declaration of Competing Interest

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