Diagnostic accuracy of the Enferplex Bovine Tuberculosis antibody test in cattle sera

Diagnostic accuracy of the Enferplex Bovine Tuberculosis antibody test in cattle sera

Amanda O'Brien[1], John Clarke[1], Alastair Hayton[2], Andy Adler[2], Keith Cutler[2], Darren J. Shaw[3], Clare Whelan[1], Neil J. Watt[4], Gordon D. Harkiss[4#]

[1]Enfer Scientific, Unit T, M7 Business Park, Newhall, Naas, County Kildare, Ireland.

[2]The Transmission Hall, Rampisham Business Centre, Rampisham Down, Maiden Newton, Dorset, DT2 0HS.

[3]Royal (Dick) School of Veterinary Studies & The Roslin Institute, University of Edinburgh, Easter Bush Campus, Edinburgh UK.

[4]MV Diagnostics Ltd, Roslin Innovation Centre, University of Edinburgh, Easter Bush Campus, Edinburgh, UK.

[#]Address correspondence to Gordon D. Harkiss, info@mvdiagnostics.co.uk

Abstract

Bovine tuberculosis (bTB) is a contagious bacterial disease of worldwide economic, zoonotic and welfare importance caused mainly by *Mycobacterium bovis* infection. Current regulatory diagnostic methods lack sensitivity and require improvement. We have developed a multiplex serological test for bovine tuberculosis and here we provide an estimate of the diagnostic accuracy of the test in cattle. Positive and negative reference serum samples were obtained from animals from Europe and the United States of America. The diagnostic specificity estimate was 98.4% and 99.7% using high sensitivity and high specificity settings of the test respectively. Tuberculin boosting did not affect the overall specificity estimate. The diagnostic sensitivity in samples from *Mycobacterium bovis* culture positive animals following tuberculin boosting was 93.9%.The relative sensitivity following boosting in tuberculin test positive, lesion positive animals and interferon gamma test positive, lesion positive animals was 97.2% and 96.9%respectively. In tuberculin test negative, lesion positive animals and in interferon gamma test negative, lesion positive animals, the relative sensitivity following tuberculin boosting was 88.2% and 83.6% respectively. The results show that the test has high diagnostic sensitivity and specificity and can detect infected animals that are missed by tuberculin and interferon gamma testing.

Introduction

Despite official control programmes, bovine tuberculosis (bTB) continues to be a serious economic, zoonotic and welfare problem worldwide. Failure to detect infected animals due to the poor sensitivity of officially approved diagnostic tests is thought to be an important contributing factor [1 – 5]. No gold standard diagnostic tests exist for bTB. Culture of *Mycobacterium bovis (M. bovis)* from post-mortem tissue is definitive proof of infection, though the sensitivity of bacterial isolation from tissues varies from 58.0% - 80.0% [6, 7]. In some countries, the presence of visible lesions (VL) typical of bTB at post-mortem is taken as evidence of *M. bovis* infection.

Tuberculin tests (TT) are approved for disclosure of infected herds and animals by the World Organisation for Animal Health (WOAH), formerly Office International des Epizooties (OIE) and the European Union (EU). Given the high specificity (99.98%) reported for the Single Intradermal Comparative Cervical Tuberculin test (SICCT) [8], a positive result in this test is regarded as very good indirect evidence of infection, though the reported sensitivity varies from 40.0% - 100% [4, 5, 9 –12]. The Single Intradermal Cervical tuberculin test (SICT) and the Caudal Fold Tuberculin test (CFT) are approved by the WOAH and used in many countries as primary screening tests. The specificity estimates for the CFT range from 89.2 – 99.0% with a sensitivity ranging between 63.2 – 93.0% [9, 13]. The SICT specificity varies between 53.1 – 99.0% [9, 14, 15]. The sensitivity estimates of the SICT range from 80.2 – 100% [9], though Bayesian analysis yielded a range from 53.0 – 69.4% [15]

The interferon gamma (IFNγ) test is WOAH validated and accepted as a supplemental test by the EU. The test has higher sensitivity estimates compared to the SICCT, ranging from 67.0% - 85.8% [4, 5, 9], but has a lower specificity (96.6%: range 85.0 – 99.6%) than the SICCT [9, 15, 16].

Recently, serology tests have been evaluated for their ability to detect infected animals [17 – 23]. These studies have shown that antibody responses can appear as early as 3 – 8 weeks post infection and challenge the view that such responses only appear in the late phases of the disease and have low sensitivity [9]. Several studies have shown that antibody responses are boosted following injection of tuberculin, with the optimum time for anamnestic responses to be detected occurring within a few days and lasting up to 30 days or longer post TT [19, 21–27]. The above studies suggest that serology tests could play a useful role in detecting and controlling bTB.

We have developed a sensitive chemiluminescent multiplex serology test – the Enferplex Bovine TB antibody test (Enferplex bTB test) - for detecting antibodies in bTB infected cattle based on an array of antigens printed onto the surface of microtitre plates. Responses to individual antigens in the Enferplex bTB test can be detected and quantified. The results of using prototype versions of the methodology have been published previously [10, 28 – 31]. Here we describe an assessment of the diagnostic performance the Enferplex bTB test using 11 target antigens with a view to assessing its possible value in aiding the control of bTB in cattle. The specific aims were to (a) determine the test sensitivity and specificity using reference samples from the EU and the USA, with and without anamnestic boosting of antibody responses by injection of partially purified derivative of bovine tuberculin (PPDb), and (b) investigate the ability of the test to detect infected animals missed by cell-mediated diagnostic tests.

Results

Estimates of diagnostic specificity and sensitivity of the Enferplex Bovine TB antibody test.

Negative and positive references samples used in the study to estimate diagnostic specificity and sensitivity are shown in Tables 1 and 2 respectively.

91 **Table 1. Summary of negative reference serum samples**

[a]Origin of samples	Number of samples	Number of herds	Age (Years) M +/- SD	[d]Reference standard
UK Set 1	1150	72	4.9 ± 2.1	England, SICCT tested every 4 years: SICCT negative > 8 years; no bTB breakdowns within 10 km in previous 12 months. 1130 MAP ELISA negative; 20 MAP ELISA positive. Non-boosted
UK Set 2	1129	14	5.6 ± 3.16	Scotland OTF status, slaughterhouse surveillance,1118 MAP ELISA negative, 11 MAP positive NON-BOOSTED.
UK Set 3	183	3	NK	England, SICCT tested every 4 years; SICCT negative > 8 years, no bTB breakdowns within 10 Km in previous 12 months. Boosted.
IE Set 1	204	37	3.8 ± 3.0	SICCT negative, IFNγ negative herds from low bTB prevalence areas MAP antibody negative; 1 MAP culture positive.
IE Set 2	439	66	[b]NK	SICCT negative, from low bTB prevalence areas. 300 MAP antibody negative ;139 MAP antibody positive.
CH/LI	554	33	[c]2.4 ± 0.6 (n= 133)	OTF country status, SICCT negative, no recent history of bTB.
NO Set 1	248	13	[c]3.9 ± 1.6 (n = 210)	OTF country status, slaughterhouse surveillance, no bTB for > 20 years, MAP antibody negative.
NO Set 2	190	8	[b]NK	OTF status, slaughterhouse surveillance, no bTB for > 20 years, 182 MAP antibody negative; 3 MAP antibody positive; 5 unknown MAP status.
USA Set 1	336	[a]NK	3.3 ± 2.0	CFT test negative from bTB free herds from 11 States. 335 MAP antibody negative; 1 MAP antibody positive;
USA Set 2	45	[a]NK	[b]NK	CFT negative from bTB free herds, 45 MAP culture positive; 43 MAP antibody positive.
NL	100	[a]NK	[b]NK	OTF country status, slaughterhouse surveillance, 100 MAP culture positive; 33 MAP antibody positive.

ES	10	[a]NK	[b]NK	OTF herd status, no recent history of bTB, slaughterhouse surveillance, MAP lesion positive at post-mortem, 10 MAP antibody positive.
FR Set 1	3	[a]NK	[b]NK	OTF country status, SICT negative, no recent history of bTB, 3 MAP culture positive.
FR Set 2	161	1	[b]NK	OTF country status, SICT negative, no recent history of bTB, Boosted.
TOTAL	**4,752**	**247**	**5.2 ± 2.8**	**Bovine TB free**

[a]Serum samples were obtained from bTB free herds in the United Kingdom (UK), Ireland (IE), Switzerland/Liechtenstein (CH/LI), Norway (NO), United States of America (USA), Netherlands (NL), Spain (ES) and France (FR). [b] Not known; [c] Age data not complete (number of animals); [d]SICCT test (UK, IE, CH/LE); CFT test (USA); SICT test (ES, FR); OTF – Officially Tuberculosis Free; MAP – *Mycobacterium avium* subsp. *paratuberculosis*

Table 2. Summary of positive field reference serum samples

[a]Origin of samples	**Number of herds**	**Age in years Mean +/- SD**	**Number of samples**				
			Comparator				
			[c]TT positive	**[c]TT inconclusive reactor**	**IFNγ positive**	**Lesion positive**	***M. bovis* culture positive**
UK	746	3.9 +/- 2.6	[d]676	[d]676	1,307	453	218
IE	550	4.2 +/- 2.7	[d]1,411	6	1,302	933	20
IT	[b]NK	[b]NK	[e]15	-	110	54	88
USA	[b]NK	4.2 +/- 3.6 (n = 113)	[f]91	-	34	156	156
Total	**1296**	**4.1 +/- 3.0**	**2193**	**682**	**2753**	**1596**	**482**

[a]Serum samples were obtained from bTB breakdown herds in the UK, IE, IT, and USA. The comparators used for validation were tuberculin tests, tuberculin test inconclusive reactors, IFNγ test, lesions at post-mortem, and culture of *M. bovis* from post-mortem tissues. The total number of samples tested using the Enferplex bTB test for each comparator is shown in the last row of the Table. [b]NK – not known. [c]TT – tuberculin test. [d]SICCT; [e]SICT; [f]CFT.

Diagnostic specificity. In non-boosted animals, the overall diagnostic specificity was 98.4% (95% CI:98.0-98.7%) and 99.7% (95% CI: 99.5-99.8%) at the high sensitivity (Hse) and high specificity (Hsp) settings of the test respectively (Table 3 and Supplementary Table S1 online). In boosted samples from bTB free herds, the diagnostic specificity was 98.8% (95% CI: 97.0-99.5%) and 99.7% (95% CI: 97.4-99.8%) at the Hse setting and Hsp setting of the test respectively.

Table 3. Diagnostic specificity estimates of the Enferplex Bovine TB antibody test.

Animal category under evaluation	Sample boost status and source	Statistical Variable	High Sensitivity setting	High Specificity setting
bTB free	Non boosted UK, IE, NO, NL, CH/LE, ES, USA	N DSp CI	4408 98.4% 98.0 – 98.7	4408 99.7% 99.5 – 99.8
bTB free	Boosted UK, FR	N DSp CI	344 98.8 97.1-99.6	344 99.1 97.4-99.7
bTB free, MAP culture positive	Non-boosted NL, IE, ES, USA	N DSp CI	257 98.4% 96.1-99.4	257 100% -
bTB free, MAP ELISA positive	Non-boosted UK, IE, NO, NL, USA	N DSp CI	251 98.4% 96.0-99.4	251 100% -

Diagnostic specificity was estimated at the high sensitivity and high specificity settings using reference sera from bTB free animals with and without tuberculin boosting and with and without MAP infection. Boosted- samples taken within anamnestic window; non-boosted – samples taken out with the anamnestic window (5-30 days post tuberculin injection). N = number of samples; DSp – diagnostic specificity; CI – 95% Confidence Interval.

To assess the diagnostic specificity in bTB-free cattle infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), samples were tested from MAP culture positive animals and MAP ELISA positive animals. The results showed that at the Hse setting, the specificity was 98.4% in both MAP culture positive and MAP ELISA positive animals respectively. The equivalent estimates obtained at the Hsp setting were both 100%. These specificity estimates are close to the specificity estimates observed in bTB free cattle, indicating that MAP infection had no significant effect on the specificity of the Enferplex bTB test. The differences in diagnostic specificity observed in samples from different countries were small (Supplementary Table S2 online).

Diagnostic sensitivity. The diagnostic sensitivity estimates obtained for the Enferplex bTB test are shown in Table 4 and Supplementary Table S1 online.

Table 4. Diagnostic sensitivity estimates of the Enferplex Bovine TB antibody test.

Animal category under evaluation	Samples and source[a]	Statistical Variable	High Sensitivity setting	High Specificity setting
M. bovis culture positive	Boosted samples UK, IE	N DSe CI	214 93.9% 89.9-96.4	214 93.9% 89.9-96.4
M. bovis culture positive	Non-boosted samples UK, USA	N DSe CI	179 76.0% 69.2-81.7	179 71.5% 64.5-77.6

M. bovis culture positive	Unknown boost status IT, IE	N DSe CI	89 80.9% 71.5-87.7	89 75.3% 65.4-83.1
M. bovis culture positive	All samples UK, IE, IT, USA	N DSe CI	482 84.9% 81.4-87.8	482 82.2% 78.5-85.3
SICCT positive	Boosted UK, IE	N RSe CI	1979 94.3% 93.1-95.2	1979 91.9% 90.6-93.0
SICCT positive or CFT positive	Non-boosted UK, IE, USA	N RSe CI	229 77.7% 71.9 – 82.6	229 72.5% 66.4 – 77.9
SICT positive	Unknown boost status IT	N RSe CI	15 73.3% 48.1 – 89.1	15 66.7% 41.7 – 84.8
SICCT, SCIT or CFT positive	All samples UK, IE, IT, USA	N RSe CI	2193 92.4% 91.2-93.4	2193 89.7% 88.4-90.9
2 x IR positive	Boosted UK	N RSe CI	401 81.1% 76.9 – 84.6	401 75.1% 70.6-79.0
2 x IR positive	Non boosted UK	N RSe CI	57 56.1% 43.3 – 68.2	57 52.6% 39.9 – 65.0
2 x IR positive	All samples UK	N RSe CI	458 78.0% 73.9-81.5	458 72.3% 68.0-76.2
1 x IR positive	Boosted UK	N RSe CI	134 85.8% 78.9-90.7	134 80.6% 73.1-86.4
1 x IR positive	Non boosted UK	N RSe CI	85 43.5% 33.5-54.1	85 40.0% 30.2-50.6
1 x IR positive	All samples UK	N RSe CI	219 69.4% 63.0-75.1	219 64.8% 58.3-70.9
IFNγ test positive	Boosted samples UK, IE	N RSe CI	1341 90.0% 88.3-91.5	1341 85.6% 83.6-87.4
IFNγ test positive	Non-boosted samples UK, USA	N RSe CI	1297 30.5% 28.0-33.0	1297 25.0% 22.7-27.4
IFNγ test positive	Unknown boost status IT, IE	N RSe CI	115 67.0% 57.9-74.9	115 64.4% 55.3-72.5

IFNγ test positive	All samples UK, IE, IT, USA	N RSe CI	2753 61.0% 59.2-62.8	2753 56.2% 54.3-58.0

Diagnostic sensitivity was estimated at the high sensitivity and high specificity settings using reference sera from animals positive by *M. bovis* culture, and relative sensitivity was similarly estimated from animals positive by SICCT, CFT, IFNγ test or SICCT inconclusive reactor (IR). [a]Boosted - samples taken within anamnestic window (5-30 days post tuberculin injection); non-boosted – samples taken out with the anamnestic window; United Kingdom (UK), Ireland (IE), Italy (IT); United States of America (USA). [b]N = number of samples; DSp – diagnostic specificity; DSe – diagnostic sensitivity; RSe – relative sensitivity; CI – 95% Confidence Interval.

*M. bovis culture comparator***.** The results show that in animals that received a tuberculin boost, the diagnostic sensitivity in *M. bovis* culture positive animals was 93.9 % (95% CI: 89.9-96.4%) and 93.9% (95% CI: 89.9-96.4%) using the Hse and Hsp settings respectively, while in non-boosted animals the sensitivity was 76.0% (95% CI: 69.2-81.7%) and 71.5% (95% CI: 64.5-77.6%) at the Hse and Hsp settings respectively. The differences between boosted and non-boosted samples were statistically significant ($P < 0.001$ in both cases). Kappa analysis using boosted samples and negative controls gave kappa values of 0.820 (95% CI: 0.782-0.857) and 0.934 (95% CI: 0.909-0.959) at the Hse and Hsp setting respectively, indicating almost perfect agreement in both cases.

SICCT comparator. The Rse of the test using samples from SICCT positive animals was significantly higher statistically at 94.3% (95% CI: 93.1-95.2%) and 91.9% (95% CI: 90.6-93.0%) using boosted samples compared with 77.7% (95% CI: 71.9-82.6%) and 72.5% (95% CI: 66.4-77.9%) in SICCT positive non-boosted samples using the Hse and Hsp settings respectively ($P < 0.001$ in both cases). Kappa analysis using boosted samples and negative controls gave kappa values of 0.932 (95% CI: 0.923-0.942) and 0.935 (95% CI: 0.925-0.945) at the Hse and Hsp setting respectively, indicating almost perfect agreement in both cases.

In animals that received a tuberculin boost and showed two consecutive SICCT inconclusive reactions (2 x IR), the Rse was 81.1% (95% CI: 76.9-84.6%) and 75.1% (95% CI: 70.6-79.0%) at the Hse and Hsp settings respectively. In non-boosted samples, the Rse was significantly lower at 56.1% (95% CI: 43.3-68.2%) and 52.6% (95% CI: 39.9-65.0%) using the Hse and Hsp setting respectively (P<0.001 in both cases). Kappa analysis using boosted samples and negative controls gave kappa values of 0.800 (95% CI: 0.769-0.832) and 0.828 (95% CI: 0.797-0.859) at the Hse and Hsp setting, indicating substantial and almost perfect agreement respectively. There were 16 animals that were VL or *M. bovis* culture positive in the 2 x IR sample set – 13 boosted samples and 3 non-boosted samples. The Enferplex bTB test was positive in 13/13 boosted samples (100%) and in 2/3 non-boosted samples (66.7%) at both the Hse and Hsp settings.

In animals that received a tuberculin boost and showed an isolated SICCT IR (1 x IR), 85.8% (95% CI: 78.9-90.7%) and 80.6% (95% CI: 73.1-86.4%) were positive at the Hse and Hsp settings respectively. In non-boosted samples, 43.5% (95% CI: 33.5-54.1%) and 40.0% (95% CI: 30.2-50.6%) were positive using the Hse and Hsp setting respectively (P<0.001 in both cases). Kappa analysis using boosted samples and negative controls gave kappa values of 0.716 (95% CI: 0.659-0.772) and 0.839 (95% CI: 0.790-0.888) at the Hse and Hse setting, indicating substantial and almost perfect agreement respectively. There were 14 animals that were VL or *M. bovis* culture positive in the 1 x IR sample set – 3 boosted samples and 11 non-boosted samples. The Enferplex bTB test was positive in 3/3 boosted samples (100%) and in 5/11 non-boosted samples (45.5%) at both the Hse and Hsp settings.

IFNγ comparator. In samples from IFNγ positive animals that received a tuberculin boost, the Rse was significantly higher at 90.0% (95% CI: 88.3-91.5%) and 85.6% (95% CI: 83.6-87.4%) compared with 30.5% (95% CI: 28.0-33.0%) and 25.0% (95% CI: 22.7-27.4%) in non-boosted animals using the Hse and Hsp settings of the test respectively (P < 0.001 in

both cases). Kappa analysis using boosted samples and negative controls gave kappa values of 0.899 (95% CI: 0.886-0.913) and 0.894 (95% CI: 0.880-0.908) at the Hse and Hse setting respectively, indicating almost perfect agreement in both cases.

Estimates of relative sensitivity of the Enferplex Bovine TB antibody test in TT positive animals with and without lesions. The Rse of the test was assessed in samples from SICCT or CFT positive animals with and without bTB lesions. Serum samples from SICCT positive animals from UK and IE, and CFT positive animals from the USA, were tested using the Hse and Hsp settings of the test. The results obtained overall and in subsets relating to whether the samples were taken during the anamnestic window or not are shown in Table 5 and Supplementary Table S1 online.

Table 5. Relative sensitivity of the Enferplex Bovine TB antibody test in TT positive animals with and without lesions.

Animal category under evaluation		Samples and source[a]	Statistical variable[b]	High Sensitivity setting	High Specificity setting
SICCT positive	bTB lesion positive	Boosted UK, IE	N RSe CI	1024 97.2% 96.0-98.0	1024 96.5% 95.2-97.5
SICCT positive or CFT positive	bTB lesion positive	Non-boosted UK, IE, USA	N RSe CI	147 86.4% 79.9-91.0	147 81.0% 73.9-86.5
SCIT positive	bTB lesion positive	Unknown boost status IT	N RSe CI	6 100% -	6 83.3% 43.7-97.0
TT positive	bTB lesion positive	All samples UK, IE, IT, USA	N RSe CI	1177 95.8% 94.5-96.8	1177 94.5% 93.0-95.7
SICCT positive	bTB lesion negative	Boosted UK, IE	N RSe CI	909 91.9% 89.1-92.8	909 86.8% 84.4-88.9
SICCT positive	bTB lesion negative	Non-boosted UK, IE	N RSe CI	68 72.1% 60.4-81.3	68 66.2% 54.3-76.3
SICT positive	bTB lesion negative	Unknown boost status	N RSe	7 57.1%	7 57.1%

		IT	CI	25.0-84.2	25.0-84.2
TT positive	bTB lesion negative	All samples UK, IE, IT	N RSe CI	984 89.5% 87.5-91.3	984 85.2% 82.8-87.2
SICCT negative	bTB lesion positive	Boosted UK, IE	N RSe CI	127 88.2% 81.4-92.7	127 81.9% 74.3-87.6
SICCT negative	bTB lesion positive	Non-boosted UK, IE, USA	N RSe CI	128 50.8% 42.2-59.3	128 44.5% 36.2-53.2
SICT negative	bTB lesion positive	Unknown boost status IT	N RSe CI	11 36.4% 15.2-64.6	11 36.4% 15.2-64.6
TT negative	bTB lesion positive	All samples UK, IE, IT, USA	N RSe CI	266 68.1% 62.2-73.4	266 62.0% 56.1-67.7

Diagnostic sensitivity was estimated at the high sensitivity and high specificity settings using reference sera from SICCT positive animals showing bTB lesions at post-mortem. [a]Boosted- samples taken within anamnestic window (5-30 days post tuberculin injection); non-boosted – samples taken out with the anamnestic window; United Kingdom (UK), Ireland (IE), Italy (IT); United States of America (USA). [b]N = number; RSe – relative sensitivity; CI – 95% Confidence Interval.

SICCT positive, lesion positive animals. The Rse observed in samples from SICCT positive animals with VL which were pre-boosted with PPDb was 97.2% (95% CI: 96.0-98.0%) and 96.5% (95% CI: 95.2-97.5%) at the Hse and Hsp settings respectively. In non-boosted samples, the equivalent figures were statistically significantly lower at 86.4% (95% CI: 79.9-91.0%) and 81.0% (95% CI: 73.9-86.5% respectively ($P < 0.001$). Kappa analysis using boosted samples and negative controls gave kappa values of 0.941 (95% CI: 0.930-0.953) and 0.970 (95% CI: 0.961-0.978) at the Hse and Hsp setting respectively, indicating almost perfect agreement in both cases.

SICCT positive, lesion negative animals. The results also show that the relative sensitivity of the test in SICCT positive boosted animals that show no visible lesions at post-mortem was 91.1% (95% CI: 89.1-92.8%) and 86.8% (95% CI: 84.4-88.9%) at the Hse and Hsp settings of the test respectively. Kappa analysis using boosted samples and negative controls gave kappa values of 0.899 (95% CI: 0.884-0.915) and 0.907 (95% CI: 0.891-0.922 at the Hse and Hsp setting respectively, indicating almost perfect agreement in both cases.

SICCT negative, lesion positive animals. Samples from animals from SICCT negative animals with lesions that were pre-boosted with PPDb had a Rse of 88.2% (95% CI: 81.4-92.7%) and 81.9% (95% CI: 74.3-87.6%) at the Hse and Hsp settings respectively. In non-boosted samples, the figures were statistically significantly lower at 50.8% (95% CI: 42.2 – 59.3) and 44.5% (95% CI: 36.2 – 53.2) respectively ($P < 0.001$ in both cases). Kappa analysis using boosted samples and negative controls gave kappa values of 0.716 (95% CI: 0.658-0.773) and 0.845 (95% CI: 0.796-0.894) at the Hse and Hsp setting, indicating substantial and almost perfect agreement respectively.

Estimates of relative sensitivity of the Enferplex Bovine TB antibody test in IFNγ test positive animals with and without visible lesions. The Rse of the test was assessed in samples from animals with and without bTB lesions that were tested using the IFNγ test. Serum samples from IFNγ tested animals from UK, IE, IT and USA were assessed using the Hse and Hsp settings of the antibody test. The results obtained overall and in subsets relating to whether the samples were taken during the anamnestic window or not are shown in Table 6 and Supplementary Table S1 online.

Table 6. Relative sensitivity of Enferplex Bovine TB antibody test in IFNγ positive animals with or without visible lesions.

Animal category under evaluation	Samples and source[a]	Statistical variable[b]	High Sensitivity setting	High Specificity setting

IFNγ positive	bTB VL positive	Boosted UK, IE	N RSe CI	352 96.9% 94.5-98.3	352 95.5% 92.7-97.2
IFNγ positive	bTB VL positive	Non-boosted UK, IE, USA	N RSe CI	166 57.8% 50.2-65.1	166 51.8% 44.3-59.3
IFNγ positive	bTB VL positive	Unknown boost status IT, USA	N RSe CI	27 74.1% 55.3-86.8	27 74.1% 55.3-86.8
IFNγ positive	bTB VL positive	All samples UK, IE, IT, USA	N RSe CI	545 83.9% 80.5-86.7	545 81.1% 77.6-84.2
IFNγ positive	bTB VL negative	Boosted UK, IE	N RSe CI	936 88.5% 86.3-90.4	936 82.9% 80.4-85.2
IFNγ positive	bTB VL negative	Non-boosted UK, IE	N RSe CI	792 32.8% 29.7-36.2	792 26.6% 23.7-29.8
IFNγ positive	bTB VL negative	Unknown boost status IT	N RSe CI	16 18.8% 6.6-43.0	16 18.8% 6.6-43.0
IFNγ positive	bTB VL negative	All samples UK, IE, IT, USA	N RSe CI	1744 62.6% 60.3-64.8	1744 56.8% 54.4-59.1
IFNγ negative	bTB VL positive	Boosted IE	N RSe CI	55 83.6% 71.7-91.2	55 74.6% 61.7-84.2
IFNγ negative	bTB VL positive	Non-boosted UK, USA	N RSe CI	21 71.4% 50.1-86.2	21 66.7% 45.4-82.8
IFNγ negative	bTB VL positive	Unknown boost status IT	N RSe CI	3 66.7% -	3 66.7% -
IFNγ negative	bTB VL positive	All samples UK, IE, IT	N RSe CI	79 79.8% 69.6-87.1	79 72.2% 61.4-80.8

Diagnostic sensitivity was estimated at the high sensitivity and high specificity settings using reference sera from IFNγ positive animals showing bTB lesions at post-mortem. [a]Boosted- samples taken within anamnestic window; non-boosted – samples taken out with the anamnestic window (5-30 days post tuberculin injection); United Kingdom (UK), Ireland (IE), Italy (IT); United States of America (USA). [b]N = number; RSe – relative sensitivity; CI – 95% Confidence Interval.

IFNγ positive, visible lesion positive animals. The results show that the Rse of the test in IFNγ test positive animals with VL that were pre-boosted with PPDb was 96.9% (95% CI: 94.5-98.3%) and 95.5% (95% CI: 92.7-97.2%) respectively using the Hse and Hsp settings of the test respectively. In non-boosted samples, the figures were statistically significantly lower at 57.8% (95% CI: 50.2-65.1%) and 51.8% (95% CI: 44.3-59.3%) respectively ($P < 0.001$ in both cases). Kappa analysis using boosted samples and negative controls gave kappa values of 0.885 (95% CI: 0.860-0.910) and 0.954 (95% CI: 0.937-0.970) at the Hse and Hsp setting, indicating almost perfect agreement respectively.

IFNγ positive, VL negative animals. In IFNγ test positive animals without lesions that were pre-boosted with PPDb the Rse was 88.5% (95% CI: 86.3-90.4%) and 82.9% (95% CI: 80.4-85.2%) using the Hse and Hsp settings respectively. In non-boosted samples, the figures were considerably lower at 32.8% (95% CI: 29.7-36.2%) and 26.6% (95% CI: 23.9-29.8%) respectively ($P< 0.0001$ in both cases). Kappa analysis using boosted samples and negative controls gave kappa values of 0.883 (95% CI: 0.866-0.900) and 0.880 (95% CI: 0.862-0.897) at the Hse and Hsp setting, indicating almost perfect agreement respectively.

IFNγ negative, VL positive animals. The Rse of the test in IFNγ test negative, lesion positive animals pre-boosted with PPDb was 83.6% (95% CI: 71.7-91.2%) and 74.6% (95% CI: 61.7-84.2%) using the Hse and Hsp settings respectively. Only 21 non-boosted samples were available for testing, precluding accurate assessment of this category of sample. Kappa analysis using boosted samples and negative controls gave kappa values of 0.530 (95% CI: 0.439-0.621) and 0.742 (95% CI: 0.650-0.834) at the Hse and Hsp setting, indicating moderate and substantial agreement respectively.

Likelihood ratio analysis. Likelihood ratio analysis was performed on Dse and Dsp data obtained using the Hse setting of the test. The results are shown in Table 7.

Table 7. Likelihood ratio (LR) for positive (LR+) and negative (LR-) boosted serum samples at the high sensitivity setting.

Sample categories	DSe 95% CI	Rse 96%CI	DSp 95% CI	LR+ 95% CI	LR- 95% CI	DOR[a] 95% CI
M. bovis culture positive/ bTB free	93.9% 89.8-97.7	-	98.4% 98.0-98.7	59.2 46.8-74.8	0.06 0.04-0.10	958 521-1761
VL positive/ bTB free	-	96.2% 95.0-97.3	98.4% 98.0-98.7	60.2 47.6-76.3	0.04 0.03-0.05	1577 1073-2317
SICCT positive/ bTB free	-	94.3% 93.1-95.3	98.4% 98.0-98.7	59.2 46.6-74.7	0.06 0.05-0.07	1011 744-1373
IFNγ positive/ bTB free	-	90.0% 88.3-91.6	98.4% 98.0-98.7	56.4 44.5-71.4	0.10 0.09-0.12	555 412-748

[a]DOR – Diagnostic odds ratio

The LR+ and LR$^-$ were 58.8 (95% CI: 46.4-74.6) and 0.06 (95% CI:0.04-0.10) respectively for boosted samples from *M. bovis* culture positive animals. The DOR was 953. Similar results were obtained from animals showing VL at post-mortem, where the LR+ and LR$^-$ were 60.2 and 0.06 respectively using boosted samples. The DOR was 1577.

In boosted samples from SICCT positive animals, the LR+ and LR- were 59.2 (95% CI: 46.6-74.7) and 0.06 (95% CI: 0.05-0.07) respectively while the DOR was 1011. The LR+ and LR- were 56.4 (95% CI: 44.5-71.4) and 0.10 (95% CI: 0.09-0.12) respectively for boosted samples from IFNγ positive animals while the DOR was 555. The test thus showed good ability to rule in infection (LR+ results) and rule out infection (LR- results), with the high DOR value indicating good overall ability to rule in and rule out infection.

Repeatability of the Enferplex Bovine TB antibody test. A negative sample, a weakly positive sample and a strongly positive sample were tested in quadruplicate within run, between runs, between days, between operators, and between 3 batches. The mean S/CO ratio values obtained following subtraction of the blank value for the negative sample within

run, between 20 runs, and between 3 batches ranged from -0.024 to 0.052. The CV observed for the weak positive and strong positive samples within wells ranged between 4.0 – 6.2%, and 1.3 – 3.3% respectively across the 11 antigens. Between operators, the S/CO CVs ranged from 3.4 – 6.9% and 1.3 – 3.8% for the weak and strong positive samples respectively. The day-to-day variation ranged between 3.6 – 6.5% and 1.3 – 3.8% for the weak and strong positive samples respectively. The variation between kit batches ranged between 3.5 – 6.9% and 1.1 – 3.5% for the weak and strong positive samples respectively. The mean values for each antigen obtained over 20 runs of the assay (over 2 days by 2 operators using 3 kit batches) did not vary more than 2 SD in 97.3% and 97.8% of results for the weak positive and strong positive samples respectively. Analysis using linear mixed-effect models showed that < 1% of the variation observed was due to the plate, kit or operator. The binary results obtained for serial batch repeatability using the two-antigen rule showed complete concordance between the Enferplex bTB test two-antigen rule result and sample category across the 3 kit batches. Overall, the within run, between run and between kit batch repeatability of the Enferplex bTB test was excellent.

Reproducibility of the Enferplex Bovine TB antibody test.

Seven negative, 7 weak positive and 7 strong positive samples were tested in duplicate using two kit batches in three independent laboratories to assess reproducibility of the Enferplex bTB test.

Analytical reproducibility: The mean S/CO ratio values obtained for the negative samples were all close to zero (Supplementary Fig 1 online). Most S/CO ratio responses (63/77) obtained with weak positive samples had CVs less than 10%. There were 14 exceptions where the %CVs were >10%. Of these, 13/14 samples were associated with responses that were below threshold for the individual antigens and would be deemed to be negative

responses for those antigens. The results show that most of the S/CO ratio responses (68/77) obtained with strong positive samples had CVs less than 10%. There were 9 exceptions where the CVs were >10%. Of these, 3/9 samples were associated with responses that were below threshold for the individual antigens and would be deemed to be negative responses for those antigens. Only 1/6 of the remaining responses had a CV >20%. The CVs observed thus tended to reflect the various levels of S/CO ratios for individual antigens such that antigens with low S/CO ratios or S/CO ratios that were below threshold tended to give higher CVs, like those observed in the negative sample panel. Linear mixed-effect models were applied with kit batch, lab, plate and sample (as random effects) to determine how much of the variation in S/CO ratio values was due to these variables. For all antigens, < 1% of the variation was due to kit, laboratory, or plate, with 99% due to the sample. The results show a high degree of analytical reproducibility between laboratories for individual responses to the 11 antigens.

Diagnostic reproducibility: The results showed complete concordance between the 3 laboratories with respect to the categorisation of the positive and negative samples. The test thus shows excellent levels of analytical and diagnostic reproducibility between laboratories.

Discussion

Work in humans with TB using whole genome arrays has indicated that the antibody response was primarily directed against around 10 recombinant antigens, all of which had been shown previously to be present in PPD [32, 33]. We have used 11 antigens that are collectively recognised by a high proportion of cattle infected with *M. bovis*. The diagnostic specificity estimated using samples from bTB free animals showed a value of 98.5% and 99.7% using the Hse and Hsp settings of the test respectively. No loss in specificity was

observed in bTB free animals after a single tuberculin injection. These results are consistent with other studies in the literature (36, 37, 38).

Relatively small differences in specificity were observed between the samples from different countries or continents, suggesting little or no interference in test performance by or environmental mycobacteria or other infections. However, no samples were available from Asia, Africa or South America to allow diagnostic specificity to be assessed more widely.

MAP infection is common in cattle and has the potential to mask bTB infection in tuberculin skin tests due to cross-reactions between MAP and bTB antigens [9, 34, 35]. Such cross-reactions could potentially affect serological assays. To assess analytical specificity, samples from bTB free animals that were MAP antibody positive or MAP culture positive, or both were analysed. The results obtained were consistent with those obtained from bTB free animals without evidence of MAP infection, showing that there was no significant cross-reaction between MAP antibodies and the Enferplex bTB test antigens. A similar lack of humoral cross-reaction was observed using the Enferplex Caprine TB antibody test in goats vaccinated against MAP [31], or when PPDa from *M. avium* is used as an antigen for serological assays in bTB infected cattle [34].

The TT and the IFNγ test are indirect tests that detect cell mediated immune responses generated by the host T cell memory cells in *M. bovis* infected animals. In a similar manner, injection of PPDb is likely to result in re-stimulation of memory T and B cells *in vivo* giving rise to boosted antibody responses typical of secondary immune responses. The Dse estimates observed for the test in *M. bovis* culture positive, TT positive, or IFNγ test positive animals thus varied depending on whether the samples were taken during the anamnestic window following PPDb boosting or not. The results described here are consistent with those described in the literature in relation to anamnestic serological responses following PPDb injection of infected animals [19, 21, 24 – 27].

352 The Dse estimates in *M. bovis* culture positive boosted animals was 93.5% at both the Hse
353 and Hsp setting. The test thus detects most truly infected animals. This is consistent with the
354 view that injection of PPDb antigen stimulates anamnestic antibody responses and induces
355 the Koch phenomenon characterised by a DTH reaction causing dissolution of granulomas
356 and release of bacterial antigens [50] resulting in additional antibody production. The reason
357 for the test not detecting the remaining 6.5% of *M. bovis* culture positive animals is not
358 known. The Koch phenomenon has not been closely studied in cattle, and its extent and
359 timescale in this species is not known. The observation that these animals showed no
360 evidence of antibody production even after boosting suggests a lack of *M. bovis* primed B cell
361 memory cells in blood despite the animals being SICCT and *M. bovis* culture positive. It is
362 recognised in human TB that granulomas which are well established, particularly if
363 circumscribed with a fibrous capsule, appear to be difficult to dissolve despite a vigorous
364 DTH reaction [50]. The bacteria in such granulomas tend to be quiescent or dormant resulting
365 in low antigen release. In cattle, acid-fast bacteria are difficult to detect in some animals
366 despite the presence of florid lesions, suggesting that the bacterial load is low and
367 consequently the level of bacterial antigen would also be low. Such bacteria often require
368 application of resuscitation techniques before the they will grow *in vitro.* This, potentially,
369 could result in some animals being *M. bovis* culture positive without showing evidence of
370 antibody production.
371 Similarly, high Rse estimates using the two sensitivity settings of the test (93.6% and 91.1%
372 respectively) were observed in SICCT positive boosted animals, though some SICCT positive
373 animals were not detected. The latter may have been due to T cell control of *M. bovis*
374 replication curtailing antigen secretion that would be required for antibody responses to be
375 generated.

376 The Rse estimates using the IFNγ test as the comparator were 90.3% and 85.9% in boosted
377 animals using the Hse and Hsp settings of the test respectively. The lower estimates obtained
378 compared to the SICCT may reflect the lower specificity of the IFNγ test [9, 12, 15, 16], such
379 that some of the IFNγ positive samples may have been false positives. Also, some animals
380 may have been in the early stages of infection when IFNγ responses could be dominant over
381 antibody responses [39, 40].
382 Animals showing inconclusive TT reactions are problematical for accurate bTB diagnosis.
383 In the UK, animals showing two consecutive inconclusive reactions in short interval testing
384 are regarded as being infected and are slaughtered. When tested in the Enferplex bTB test, a
385 high proportion of both 2 x IR and 1 x IR animals gave positive reactions, giving confidence
386 that these animals were infected with *M. bovis*. The test was positive in all VL/*M. bovis*
387 culture positive boosted 2 x IR and 1 x IR animals. The results indicate that the Enferplex
388 bTB test detects SICCT inconclusive infected animals which in the case of 1 x IR animals are
389 currently not being removed to slaughter. The results suggest that the test could be useful in
390 resolving the infection status of IR animals, thereby removing SICCT or IFNγ negative
391 infected animals earlier and improving the efficiency of bTB control and eradication.
392 The diagnostic accuracy of the test was also assessed in relation to the presence or absence
393 of lesions following post-mortem using the Hse and Hsp test settings. High Rse and Dsp
394 estimates of 97.1% and 96.3% respectively were observed using samples from TT positive,
395 bTB lesion positive boosted animals. Strong correlations between antibody levels and the
396 presence of lesions in post-mortem tissues have been described in the literature [9, 39, 40]
397 and the results are thus consistent with these reports.
398 Antibody positive animals were also found in TT positive animals with no VL, an
399 observation not unexpected since lesions can be missed at post-mortem examination with
400 typically only a single VL being found in herds subject to eradication schemes [41]. For

example, the vast majority (84.7%) of the UK animals with VL had only a single VL [APHA bTB database]. It is even more difficult to detect histological lesions in VL negative animals due to the lack of information of where to sample in the tissues. Nevertheless, histological lesions, positive *M. bovis* cultures, and/or positive PCR reactions have been described in 10 – 47% of animals with no VL [42 – 44]. The positive antibody results found in this study provide confirmatory evidence that the TT positive animals without lesions were infected with *M. bovis*. Also, animals without lesions have been shown to shed *M. bovis* following experimental infection [45, 46] suggesting that they are epidemiologically important and a significant risk to the rest of the herd.

In addition, the antibody test detected 88.2% of TT negative, lesion positive boosted animals. Similar results were obtained in IFNγ test negative, lesion positive animals, where 83.6% of boosted animals were antibody positive. These results are likely to reflect the variable sensitivity of the SICCT (51 – 85% for standard interpretation) and IFNγ test (67.0 – 85.8%) reported in the literature [5]. In chronically infected herds, latent class analysis gave sensitivities for standard interpretation of SICCT between 40.5 – 57.7% [4]. This low sensitivity may reflect the development of a state of anergy in advanced disease [47]. The results thus show that the Enferplex bTB test can detect infected animals missed by the TT or IFNγ test. This is not surprising since reciprocal cell mediated and humoral responses have been documented [38, 39], and may reflect swings between type 1 and type 2 T helper cell responses during infection [39]. However, relatively few samples of SICCT negative, VL positive and IFNγ test negative, VL positive animals were available and larger sample sets are required to give a more accurate estimate of diagnostic sensitivity in these categories of infected animals. A further limitation was the lack of samples from outside the EU and the USA which will be required to determine the results found here can be generalised. The Enferplex bTB test results obtained from *M. bovis* culture positive, SICCT positive or IFNγ

positive animals underwent likelihood ratio analysis to assess the strength of the evidence they provided for *M. bovis* infection being either present or absent [48, 49]. The Enferplex bTB test showed LR results provide good diagnostic evidence of the infection being either present (LR+ 58.8 for culture positives: LR+ 60.2 for VL) or absent (LR- 0.06 in both cases for samples from bTB negative animals), DOR was 953 and 1577 respectively. These results compare with LR^+ = 32.3 (95% CI: 25.4 – 40.9); LR^- = 0.361 (5% CI: 0.314 0.412); DOR 89.4 for the IDEXX test, and LR^+ = 32.5 (95% CI: 6.6 – 125.7); LR^- = 0.158 (95% CI: 0.088 – 0.285); and DOR 205.8 for Bovigam test [49].

Overall, the results indicate that the Enferplex bTB test at the Hse or Hsp setting could be used to detect antibodies to *M. bovis* in serum from bTB-infected cattle to aid eradication and/or in surveillance schemes to detect primary infection in conjunction with other methods. For example, the test could be used to confirm a diagnosis of bTB in suspect cases, detect bTB infected animals missed by SICCT or IFNγ tests, or to aid resolving infection status of animals showing an inconclusive reaction in a TT test. Other potential uses of the test include pre- and post-movement testing, or in serological surveys to estimate prevalence of infection. In addition, the test could provide a valuable tool for slaughterhouse surveillance. However, it should be noted that the test has only been assessed in samples from European countries and the USA and that further studies are required to determine Dse and Dsp in other continents with different prevalence and *M. bovis* clades.

Methods

Study design. The study aimed to determine diagnostic sensitivity and specificity by testing defined positive and negative reference sera from animals known to be infected with *M. bovis* or known to be from herds free of bTB respectively. All methods were performed in accordance with the relevant national and international guidelines and regulations and

complied with ARRIVE guidelines. Reference sera from animals naturally infected with *M. bovis* and bTB free animals over 6 months of age from the EU and USA were tested. The positive reference sera were obtained from bTB infected animals defined by culture of *M. bovis* from post-mortem tissues (Definitive Positive Reference Standard), and by results obtained in tuberculin tests (SICCT/CFT) and the IFNγ test (Comparative Positive Reference Standards). The negative reference sera were obtained from herds that were negative in SICCT/CFT, and/or were from countries or regions that held Officially Tuberculosis Free (OTF) status and had no recent history of bTB or contact with animals likely to have been exposed to *M. bovis*. Relevant subsets of animals with bTB were also analysed. These included animals positive by TT or IFNγ tests with or without visible lesions at post-mortem. Samples taken 5 – 30 days post TT were regarded as being 'boosted' for antibody, while those taken outside this anamnestic window were regarded as being 'non-boosted'. The samples obtained encompassed wide variations in geographical location, cattle breed, age, husbandry, farm practices, and farm management. Antibodies to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) were measured using the ID.vet ID Screen Paratuberculosis Indirect ELISA or IDEXX MAP antibody test.

Reference samples.

Negative reference serum samples.

Serum samples from negative reference animals (n = 4,747) were obtained from the United Kingdom (UK), Ireland (IE), Netherlands (NL), Switzerland and Liechtenstein (CH/LI), Norway (NO), Spain (ES), France (FR) and the USA, summarised in Table 1. Samples were obtained retrospectively as remnants from serum banks held by regulatory authorities or various institutions following routine diagnostic testing from animals tested for non-

475 tuberculosis diseases (MAP, BVDV, IBR, FH, BCV, BRSV) in several counties (UK, IE,
476 NL, NO, ES, CH/LI). These samples and data were collected by local veterinary surgeons for
477 routine diagnostic purposes which is regulated by the national and EU legislation.

478 Most of samples were from females (3012/3183 gender records, 94.6%). There were 40
479 breeds/crosses included in the study. The samples were from herds in low-risk areas or
480 countries with OTF status that had been free of bTB for several years as based on routine
481 surveillance screening by SICCT, CFT, IFNγ test or post-mortem examination. In the case of
482 samples from the UK, herds had been free of bTB for at least 8 years, and no herds within 10
483 Km had recorded bTB in the previous 12 months. A further set of 339 sera from 5 herds
484 sampled 5 - 30 days after the SICCT from bTB free herds in the UK and FR were available as
485 anamnestic specificity controls. Samples from IE were obtained from herds in the low cattle
486 bTB prevalence areas defined as those herds with 2 or fewer standard SICCT reactors in the
487 annual round of skin testing over the previous preceding 5 years (Greenfield sites) [51].

488 Animals from low-risk areas or from OTF countries that were MAP culture and/or MAP
489 antibody positive were included in the negative reference set. The blood samples were taken
490 at the time of reading the TT, and therefore were “non-boosted”. The details are shown in
491 Table 1.

492 ***Positive reference serum samples.***

493 Most positive references samples from bTB infected animals were obtained retrospectively as
494 remnants from serum banks held by regulatory authorities (UK Animal and Plant Health
495 Agency, the United States Department of Agriculture, the Irish Department of Agriculture
496 and Marine, and the IZLER Biobank, Instituto Zooprofilattico Sperimentale della Lombardia
497 e dell’Emilia, IT). Some positive reference samples were obtained following regulatory
498 approval at the abattoir post slaughter.

499 The majority of samples were from females (3333/3979 gender records; 83.7%). There were
500 40 breeds/crosses included in the study. The animals were positive by one or more of the
501 following criteria: SICCT, SICT, CFT, IFNγ test, VL, or *M. bovis* culture. Sera from animals
502 showing two consecutive inconclusive reactions in the SICCT (2 x IR) were included as
503 positive reference samples (APHA Official Veterinarian instructions available from:
504 http://apha.defra.gov.uk/External_OV_Instructions/TB_Instructions/Skin_Test/Skin_Test_Da
505 y_Two.html). Animals showing a single IR reaction were also included for comparative
506 purposes. Typically, some of these animals go on to show an IR reaction at the next short
507 interval test and become a 2 x IR category animal. Of 131 UK SICTT positive animals with
508 VL, 111 (84.7%) registered a single VL, 17 (13.0%) had 2 VLs and 3 (2.6%) had 3 VLs
509 (APHA bTB database).
510 Samples were taken approximately 5 – 30 days post PPD injection when anamnestic antibody
511 responses were likely to be developing or optimal (termed 'boosted') or outside this time
512 frame when anamnestic responses were unlikely to be present (termed 'non-boosted'). The
513 details are summarised in Table 2.
514 In this study, a total of 4,747 negative reference samples were used to determine test
515 specificity, while a total of 4,718 positive reference samples were used to determine test
516 sensitivity: 482 *M. bovis* culture animals, 2,651 TT positive animals, and 2,753 IFNγ positive
517 animals were used to determine test sensitivity.

518 Enferplex Bovine TB antibody test description

519 The antigens used in the study were as follows: Rv2975 synthetic peptide p6 [52]; PPDb;
520 recombinant Rv2873; recombinant Rv2975; Bovine TB cocktail; recombinant Rv2031c-
521 Rv1886c fusion protein; recombinant Rv3875-Rv3874 fusion protein; recombinant Rv3874-
522 Rv3875 fusion protein; recombinant Rv2626; recombinant Rv0251c; recombinant Rv2031c.

Rv2975 peptide p6 and PPDb were obtained from Genosphere Biotechnologies, Paris, France and Prionics Ag, Switzerland respectively, while the other antigens were obtained from Lionex (Braunschweig, Germany) and Genscript Biotech (Netherlands). These antigens were chosen on the basis that they give high operational specificity using the two-antigen rule while individually providing incremental additions to the overall Dse sensitivity of the test. The antigens were deposited in a multiplex planar array as individual 30 nl spots into wells of 96 well black polystyrene microtiter plates using BioDot aspirate/dispense platforms. Plates were blocked, stabilised, dried and stored at 2-8ºC until use. The assay was carried out as previously described [28, 31], with the following modifications. Serum samples were diluted 1:200 into sample dilution buffer (Enfer Buffer B, Enfer Scientific) and mixed before 50ul was added per well. The plates were incubated at 37ºC for 60 min with agitation (900 rpm). The plates were washed 6 times with 1X Wash (Enfer Wash buffer, Enfer Scientific) and aspirated. The detection antibody, sheep anti-bovine IgG - HRP (Bethyl Laboratories) was prepared to 1:20000 dilution. The plates were incubated at 37ºC for 60 min with agitation (900 rpm). The plates were washed as above and 50ul of prepared chemiluminescent substrate (50:50 dilution of substrate and diluent), (Advasta, USA) was added per well. Relative light units (RLU) were captured (220 second exposure) immediately, using Quansys Biosciences Q-View™ LS imager and Q-View™ software (v 3.1). The results were defined using the Enferplex Bovine TB Macro, based on individual antigen thresholds after subtracting the RLU value obtained from a blank spot. The individual antigen thresholds were set using known positive and negative serum samples. Individual antibody levels were determined by calculating the signal/cut-off (S/CO) ratio for each antigen. The performance levels indicated in this study were based on multiple batches of the Enferplex bTB test and reflect the biological diversity with respect to kit components (antigens, buffers, conjugates, positive and negative controls). The thresholds for individual

548 antigens in the Enferplex bTB test were selected empirically. Two threshold settings were
549 established by selecting individual antigen cut-offs appropriately to give a Hse setting with a
550 target specificity of 98.0%, which optimises the test for eradication purposes and a Hsp
551 setting with a target specificity of 99.5%, which optimises the test for screening purposes.
552 Thus, the higher the sensitivity the lower the specificity and vice versa.
553 The threshold for overall assay positivity was set based on a two - antigen rule, whereby the
554 RLU signals from two or more antigens need to be above their individual antigen thresholds
555 for the sample to be registered as "positive" [28]. Thresholds for individual antigens were set
556 between 95.1 – 99.8% specificity based on true negative reference sera to give the "Hse"
557 setting using the two-antigen rule. Thresholds for individual antigens were set between 97.3 –
558 99.9% specificity based on true negative reference sera to give the "Hsp" setting for the test
559 using the two-antigen rule. An 'Enferplex Bovine TBMacro', automatically calculates the
560 results for Hse and Hsp.
561 **Repeatability and reproducibility trials.** To determine the within-run, between-run,
562 and between-batch variation, three categories of sera were used to test within run
563 repeatability: one serum sample that was negative against all 11 antigens; one serum dilution
564 for each antigen giving strong positivity; one serum dilution for each antigen giving weak
565 positivity. The samples were run in quadruplicate over 20 runs, split between two days and
566 two operators for three kit batches. For reproducibility studies, an evaluation panel of serum
567 samples comprising negative, weak positive and strong positive serum samples were blinded
568 and sent to two independent laboratories for analytical reproducibility testing. Seven negative
569 samples, seven weak positive samples, and seven strong positive samples (based on the two-
570 antigen rule) were tested blind in duplicate using two plates from two different kit batches
571 and one technician in the Enfer laboratory (Enfer Scientific laboratory, IE) and each of the
572 two independent laboratories: Animal and Plant Health Agency (APHA), Weybridge, UK;

573 Molde Mastitis Reference Laboratory, TINE, NO. The results from the Molde and
574 Weybridge laboratories were sent to Enfer Scientific for un-blinding and analysis.
575 Data were expressed as mean, SD, and 95% Confidence interval. Differences in proportions
576 were assessed using Fisher's Exact test. The degree of agreement between Enferplex test and
577 positive and negative reference comparators was assessed using Cohen's Kappa analysis.
578 Likelihood ratios were calculated using MedCalc statistical programme. For the repeatability
579 and reproducibility analyses, a series of linear mixed-effect models were run with kit batch,
580 laboratory, microtitre plate and sample were entered as random effects. These models also
581 permitted the amount of variation explained by batch, laboratory, microtitre plate and sample
582 to be assessed via calculation of intra-class correlation coefficients (ICCs). The results
583 included the overall means, SD, coefficient of variation (CV) defined as the ratio of the SD to
584 the mean expressed as a percentage, and 95% CI and an estimate of how much variation was
585 due to these variables. The analyses were carried out in R (v3.51, (C) 2018 The R Foundation
586 for Statistical Computing), using the lme4 (v1.1-18.1), lmerTest (v3.0-1) and sjPlot (v2.6.0)
587 and sjstats (v0.17.0) packages.
588

589 # References

590 1. Humblet, M-F., Boschiroli, M.L., Saegerman, C. Classification of worldwide bovine tuberculosis
591 risk factors in cattle: a stratified approach. *Vet. Res*. **40**, 50-74 (2009).
592 2. Álvarez, J. *et al*. Risk factors associated with negative *in-vivo* diagnostic results in bovine
593 tuberculosis-infected cattle in Spain. *BMC Vet. Res.***10**, 14-23 (2014).
594 3. Brooks-Pollock, H., Roberts, G.O., Keeling, M.J. A dynamic model of bovine tuberculosis
595 spread and control in Great Britain. *Nature* **511(7508**), 228-231(2014).
596 4. Lahuerta-Marina, A. *et al*. Bayesian latent class estimation of sensitivity and specificity
597 parameters of diagnostic tests for bovine tuberculosis in chronically infected herds in Northern
598 Ireland. *Vet. J*. **238**,15–21(2018).

5. Nunez-Garcia, J. *et al*. Meta-analyses of the sensitivity and specificity of ante-mortem and post-mortem diagnostic tests for bovine tuberculosis in the UK and Ireland. *Prev. Vet. Med*. **153**, 94–107 (2018).
6. Corner, L.A.L., Gormley, E., Pfeiffer, E.D.U. Primary isolation of *Mycobacterium bovis* from bovine tissues: Conditions for maximising the number of positive cultures. *Vet. Microbiol*. **156**, 162–171 (2012).
7. Courcoul, A. *et al*. Estimation of sensitivity and specificity of bacteriology, histopathology and PCR for the confirmatory diagnosis of bovine tuberculosis using latent class analysis. PLOS ONE **9(3)**, e90334 (2014).
8. Goodchild, A.V., Downs, S.H., Upton, P., Wood, J.L.N., de la Rua-Domenech, R. Specificity of the comparative skin test for bovine tuberculosis in Great Britain. *Vet. Rec*. **177(10),** 258-266 (2015).
9. de la Rua-Domenech, R. *et al*. Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, γ interferon assay and other ancillary diagnostic techniques. *Res. Vet. Sci*. **81,** 190-210 (2006).
10. Clegg, T.A. *et al*. Using latent class analysis to estimate the test characteristics of the γ-interferon test, the single intradermal comparative tuberculin test and a multiplex immunoassay under Irish conditions. *Vet. Microbiol*. **151**, 68–76 (2011).
11. Costello, E., Egan, J.W.A., Quigley, F.C., O'Reilly, P.F. Performance of the single intradermal comparative tuberculin test in identifying cattle with tuberculous lesions in Irish herds. *Vet. Rec*. **141**, 222-224 (1997).
12. Gormley, E. *et al*. Identification of risk factors associated with disclosure of false positive bovine tuberculosis reactors using the gamma-interferon (IFNγ) assay. *Vet. Res*. **44**,117-125 (2013).
13. Farnham, M.W., Norby, B., Goldsmith, T.J., Wells, S.J. Meta-analysis of field studies on bovine tuberculosis skin tests in United States cattle herds. *Prev. Vet. Med.* **103(2)**, 234-242 (2012).

14. Schiller, I. *et al.* Bovine tuberculosis. A review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transbound. Emerg. Dis*. **57**, 205–220 (2010).
15. Alvarez, J. *et al.* Evaluation of the sensitivity and specificity of bovine tuberculosis diagnostic tests in naturally infected cattle herds using a Bayesian approach. *Vet. Microbiol*. **155(1)**, 38-43 (2012).
16. Antognoli, M.C. *et al*. Analysis of the diagnostic accuracy of the gamma interferon assay for detection of bovine tuberculosis in U.S. herds. *Prev. Vet. Med.* **101**, 35– 41 (2011).
17. Lyashchenko, K. *et al*. Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis. Infect. Immun*. **75(5)**,2462–2467 (2004).
18. Koo, D.C. *et al*. Use of rMPB70 protein and ESAT-6 peptide as antigens for comparison of the enzyme-linked immunosorbent, immunochromatographic, and latex bead agglutination assays for serodiagnosis of bovine tuberculosis. *J. Clin. Microbiol.* **43(9)**, 4498-506 (2005).
19. Waters, W.R. *et al*. Early antibody responses to experimental *Mycobacterium bovis* infection of cattle. *Clin. Vaccine Immunol*. **13**, 648-654 (2006).
20. Green, L.R. *et al.* Single-antigen serological testing for bovine tuberculosis. *Clin. Vaccine Immunol.* **16(9)**, 1309–1313 (2009).
21. Waters, W.R. *et al*, Development and evaluation of an enzyme-linked immunosorbent assay for use in the detection of bovine tuberculosis in cattle. *Clin. Vaccine Immunol*. **18**, 1882–1888 **(**2011).
22. Casal, C, *et al*. Strategic use of serology for the diagnosis of bovine tuberculosis after intradermal skin testing. *Vet. Microbiol.* **170**, 342–351 (2014).
23. Casal, C. *et al.* Antibody detection tests improve the sensitivity of tuberculosis diagnosis in cattle. *Res. Vet. Sci*. **112**, 214–221 (2017).
24. Lightbody, K.A., Skuce, R.A., Neill, S.D., Pollock, J.M. Mycobacterial antigen-specific antibody responses in bovine tuberculosis: an ELISA with potential to confirm disease status. *Vet. Rec.* **142**, 295-300 (1998).

25. Thom, M. *et al*. The effect of repeated tuberculin skin testing of cattle on immune responses and disease following experimental infection with *Mycobacterium bovis*. *Vet. Immunol. Immunopathol.* 102:399–412 (2004).
26. Villarreal-Ramos, B. *et al*. Influence of the nature of the antigen on the boosting of responses to mycobacteria in *M. bovis*-BCG vaccinated cattle. *Vaccine* **24**, 6850–6858 (2006).
27. Waters, W.R. *et al.* Effects of serial skin testing with purified protein derivative on the level and quality of antibodies to complex and defined antigens in *Mycobacterium bovis*-infected cattle. *Clin. Vaccine Immunol.* **22**, 641-649 (2015).
28. Whelan, C. *et al.* Multiplex immunoassay for serological diagnosis of *Mycobacterium bovis* infection in cattle. *Clin. Vaccine Immunol*. **15**, 1834-1838 (2008).
29. Whelan, C. *et al*. Performance of the Enferplex TB Assay with cattle in Great Britain and assessment of its suitability as a test to distinguish infected and vaccinated animals. *Clin. Vaccine Immunol*. **17**, 813-817 (2010).
30. Whelan, C. *et al*. Use of a multiplex enzyme-linked immunosorbent assay to detect a subpopulation of *Mycobacterium bovis*–infected animals deemed negative or inconclusive by the single intradermal comparative tuberculin skin test. *J. Vet. Diagn. Invest*. **23**, 499-503 (2011).
31. O'Brien, A. *et al*. Serological analysis of tuberculosis in goats by use of the Enferplex Caprine TB multiplex test. *Clin. Vaccine Immunol*. **24**, e00518-16 (2017).
32. Kunnath-Velayudhan, S. *et al*. Dynamic antibody responses to the *Mycobacterium tuberculosis* proteome. *Proc. Natl. Acad. Sci. USA* **107**, 14703–14708 (2010).
33. Kunnath-Velayudhan, S. *et al.* Proteome-Scale antibody responses and outcome of *mycobacterium tuberculosis* infection in nonhuman primates and in tuberculosis patients. *J. Infect. Dis*. **206**, 697–705 (2012).
34. Aranaz, A. *et al*. Assessment of diagnostic tools for eradication of bovine tuberculosis in cattle co-infected with *Mycobacterium bovis* and *M. avium* subsp. *paratuberculosis*. *Vet. Res*. **37**, 593–606 (2006).
35. Alvarez, J. *et al*. Interference of paratuberculosis with the diagnosis of tuberculosis in a goat flock with a natural mixed infection. *Vet. Microbiol.* **128**, 72–80 (2008).

680 36. Thom, M.L. *et al*. The effect of tuberculin testing on the development of cell-mediated immune
681 responses during *Mycobacterium bovis* infection. *Vet. Immunol. Immunopathol*. **114**, 25–36
682 (2006).
683 37. Palmer, M.V. *et al*. Effects of Different tuberculin skin-testing regimens on gamma interferon
684 and antibody responses in cattle experimentally infected with *Mycobacterium bovis. Clin.*
685 *Vaccine Immunol.* **13(3)**, 387–394 (2006).
686 38. Che-Amat, A. *et al*. Effects of repeated comparative intradermal tuberculin testing on test results:
687 a longitudinal study in TB-free red deer. *BMC Vet. Res.* 12. 184.
688 39. Ritacco, V. *et al*. Reciprocal cellular and humoral immune responses in bovine tuberculosis. *Res.*
689 *Vet. Sci.* **50(3)**, 365-567 (1991).
690 40. Welsh, M.D. *et al*. JM Influence of pathological progression on the balance between cellular and
691 humoral immune responses in bovine tuberculosis. *Immunol*. **114**, 101–111 (2005).
692 41. Corner, L.A. *et al*. Efficiency of inspection procedures for the detection of tuberculous lesions in
693 cattle. *Aust. Vet. J.* **61**, 389-392 (1990).
694 42. Corner, L.A. Post mortem diagnosis of *Mycobacterium bovis* infection in cattle. *Vet. Microbiol*.
695 **40**, 53-63 (1994).
696 43. Liebana, E. *et al*. Pathology of naturally occurring bovine tuberculosis in England and Wales.
697 *Vet. J.* **176**, 354–360 (2008).
698 44. Araújo, C.P. *et al*. Direct detection of *Mycobacterium tuberculosis* complex in bovine and
699 bubaline tissues through nested-PCR. *Br. J. Microbiol*. **45 (2)**, 633-640 (2014).
700 45. McCorry, T. *et al*. Shedding of *Mycobacterium bovis* in the nasal mucus of cattle infected
701 experimentally with tuberculosis by the intranasal and intratracheal routes. *Vet. Rec*. **157**, 613-
702 618 (2005).
703 46. Pollock, J.M., Neill, S.D. *Mycobacterium bovis* infection and tuberculosis in cattle. *Vet. J.* **163**,
704 115-127 (2002).
705 47. Lepper, A.W.D., Pearson, C.W., Corner, L.A. Anergy to tuberculin in beef cattle. *Austr. Vet. J.*
706 **53**, 214-216 (1977).

48. Hayden, S.R., Brown, M.D. Likelihood ratio: a powerful tool for incorporating the results of a diagnostic test into clinical decision making. *Ann. Emerg. Med*. **33(5),** 575-580 (1999).
49. Caraguel, C.G.B., Colling, A. Diagnostic likelihood ratio – the next generation of diagnostic test accuracy measurement. *Rev. Sci. Tech. Off. Int. Epiz*. **40(1),** 299-309 (2021).
50. Hunter, R.L. The Pathogenesis of Tuberculosis–The Koch Phenomenon Reinstated. *Pathogens* **9,** 813- 826 (2020).
51. Murphy, D. *et. al*. Tuberculosis in cattle herds are sentinels for *Mycobacterium bovis* infection in European badgers (Mele meles): the Irish Greenfield study. *Vet. Microbiol*. **151 (1-2),** 120-125 (2011).
52. Lightbody, K.A., McNair, J., Neill, S.D., Pollock, J.M. IgG isotype antibody responses to epitopes of the *Mycobacterium bovis* protein MPB70 in immunised and in tuberculin skin test-reactor cattle. *Vet. Microbiol.* **75:** 177-188 (2000).

Acknowledgements

We would like to thank the following for supplying reference serum samples and associated data: Dr. Liv Solverod, Mastitis Reference Laboratory, TINE, Molde, Norway; Dr. Tore Tollersrud, Norwegian Veterinary Institute, Oslo, Norway; Dr. Ramon Juste, NEIKER-Tecnalia: Derio, Bizkaia, Spain; Dr Kees van Maanen, GD, Deventer, Holland; Professor. Martin Vordermeier, Animal and Plant Health Agency (APHA), Addlestone, UK; Paul Upton, APHA, Addlestone, UK; James Tiller, APHA, Addlestone, UK; Dr George Caldow, Scottish Rural University College, Edinburgh, UK; Rhiannon Purbrick, Synergy Farm Health, Evershot, UK; Dr Kevin Kenny, Department of Agriculture, Food and Marine, Celbridge, Co. Kildare, Ireland; Dr Noel Byrne, Moorepark Food and Research Centre, Teagasc, Fermoy, Co. Cork, Ireland; Professor Irene Schiller, University of Berne, Switzerland; Dr Maria Pacciarini, Instituto Zooprofilattico Sperimentale della Lombardia e

dell'Emilia (IZSLER), Brescia, Italy; Dr Valentín Pérez, Dpto. Sanidad Animal (Anatomía Patológica), Facultad de Veterinaria, Universidad de León, Spain.

Author Contributions

G.H., N.W., A.O. and A.H. conceived and designed the experiments. AH, AA, KC, NW and AO sourced the reference samples. A.O. and J.C. performed the laboratory experiments. G.H., A.O., N.W. analysed and interpreted the diagnostic data. D.S. analysed and interpreted the repeatability and reproducibility data. G.H. outlined the manuscript. G.H., A.O., N.W., A.H., A.A., KC, C.W. and D.S. wrote manuscript. All authors read and approved the final manuscript.

Funding

This research received no specific grant from any funding agency in the public or not-for-profit sectors. The work was funded internally by the three commercial companies involved.

Competing Interests

A.O., J.C., C.W. (Enfer Scientific), A.H, A.A, K.C. (SureFarm Ltd), and G.H., N.W. (MV Diagnostics Ltd.) declare commercial interests in the Enferplex Bovine TB antibody test. D.S. (University of Edinburgh) declares no competing interests.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary files.

Additional information

Supplementary information for this paper is available in supplementary files.

Correspondence and requests for materials should be addressed to G.H.