Supplementary Materials

Anti- phenolic glycolipid antibodies in *Mycobacterium bovis* infected cattle

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Supplementary Table S1: Characteristics of M. bovis-infected cattle cohort

Sample No.	Gender	Breed code	Age	Lesion location: Head (H), Lung (L), alimentary tract (A)	Laboratory culture of *M. bovis*	Typing information of *M. bovis*	SICCT
1	Female	LIMX	1 year	L	positive	Spoligo type9	positive
2	Male	SAX	1 year 7 months	H	positive	Spoligo type9	positive
3	Male	LIMX	2 years 2 months	H	positive	Spoligo type9	positive
4	Male	LIMX	2 years 3 months	H	positive	Spoligo type9	positive
5	Male	LIMX	2 years 3 months	H	positive	Spoligo type9	positive
6	Male	LIMX	2 years 2 months	H	positive	Spoligo type9	positive
7	Female	BBX	1 year 2 months	H	positive	Spoligo type9	positive
8	Male	LIMX	1 year	H	positive	Spoligo type9	positive
9	Male	LIMX	2 years 4 months	H	positive	Spoligo type9	positive
10	Male	LIMX	2 years	H	positive	Spoligo type9	positive
11	Male	LIMX	2 years 1 month	H	positive	Spoligo type9	positive
12	Female	FR	10 years 2 months	L	positive	Spoligo type17	positive
13	Male	LIMX	2 years 5 months	H	positive	Spoligo type9	positive
14	Male	CHX	2 years	H	positive	Spoligo type9	positive
15	Male	LIMX	1 year 7 months	H	positive	Spoligo type9	positive
16	Male	CHX	2 years 3 months	H	positive	Spoligo type9	positive
17	Female	DEX	1 years 3 months	A	positive	Spoligo type17	positive
18	Male	WB	2 years 3 months	H, L & A	positive	na	positive
19	Male	LIMX	2 years 4 months	H	positive	Spoligo type9	positive
20	Female	HF	4 years 9 months	H, L & A	positive	Spoligo type9	positive
21	Male	CHX	2 years	H	positive	Spoligo type9	positive
22	Male	LIMX	2 years	H, L & A	positive	Spoligo type9	positive
23	Male	LIMX	2 years 1 month	H, L & A	positive	Spoligo type9	positive
24	Male	HO	3 years 1 month	H, L & A	positive	Spoligo type9	positive
25	Male	LIMX	2 years	H	positive	Spoligo type9	positive
26	Male	HF	1 year 4 months	H	positive	Spoligo type9	positive
27	Male	CHX	2 years 2 months	H, L & A	positive	Spoligo type9	positive
28	Female	BH	3 years	H, L & A	positive	Spoligo type9	positive
29	Female	HF	4 years 3 months	H, L & A	positive	Spoligo type9	positive
30	Female	HF	3 years	H	positive	Spoligo type9	positive
31	Female	HO	5 years	H, L & A	positive	na	positive
32	Female	HO	9 years	H	positive	na	positive
33	Female	HO	4 years	H	positive	Spoligo type9	positive
34	Male	HF	1 year	H, L & A	positive	Spoligo type17	positive
35	Female	LIMX	2 years	H, L & A	positive	Spoligo type9	positive
36	Female	LIMX	1 year 9 months	H, L & A	positive	Spoligo type9	positive
37	Male	HF	2 years	H	positive	Spoligo type9	positive
38	Female	LIMX	2 years	H, L & A	positive	Spoligo type9	positive
39	Male	HF	1 year	H, L & A	positive	Spoligo type17	positive
40	Female	HX	12 years 7 months	H, L & A	positive	Spoligo type9	positive

41	Male	LIMX	2 years	H, L & A	positive	na	positive
42	Female	HF	4 years 7 months	L	positive	Spoligo type9	positive
43	Female	SM	1 year 4 months	L	positive	Spoligo type17	positive
44	Female	HF	2 years 9 months	L	positive	Spoligo type9	positive
45	Female	HEX	2 years	H	positive	Spoligo type9	positive
46	Female	HEX	1 year 3 months	H	positive	Spoligo type9	positive
47	Female	HEX	1 year 3 months	L	positive	na	positive
48	Female	HEX	2 years 3 months	H & L	positive	Spoligo type9	positive
49	Female	BBX	1 year 7 months	L	positive	Spoligo type9	positive
50	Female	BBX	1 year 7 months	L	positive	Spoligo type9	positive
51	na	na	na	na	positive	Spoligo type9	positive
52	na	na	na	na	positive	Spoligo type9	positive
53	Female	LIM	1 year 4 months	H	positive	Spoligo type9	positive
54	Male	WBX	2 years 4 months	L	positive	Spoligo type9	positive
55	na	na	10 years 7 months	A	positive	Spoligo type9	positive
56	Female	LIM	1 year 9 months	H	positive	Spoligo type9	positive
57	Female	SMX	2 years 3 months	L	positive	Spoligo type9	positive
58	na	na	10 years 11 months	A	positive	Spoligo type9	positive
59	Female	LIMX	1 year 1 month	A	positive	Spoligo type17	positive
60	na	na	1 year 9 months	A	positive	Spoligo type9	positive
61	Female	CHX	1 year 11 months	L	positive	Spoligo type9	positive
62	Male	LIMX	2 years	L	positive	Spoligo type9	positive
63	Female	HEX	2 years	L	positive	Spoligo type9	positive
64	Male	LIMX	2 years	L	positive	Spoligo type9	positive
65	Female	CHX	2 years	L	positive	Spoligo type9	positive
66	Male	CHX	2 years	L	positive	Spoligo type9	positive
67	Female	HEX	1 year 10 months	A	positive	Spoligo type9	positive
68	Female	CHX	1 year 11 months	L	positive	Spoligo type9	positive
69	Male	HEX	2 years	L	positive	Spoligo type9	positive
70	Male	LIMX	2years	L	positive	Spoligo type9	positive
71	Female	CHX	2 years	A	positive	Spoligo type9	positive
72	Male	CHX	2 years	L	positive	Spoligo type9	positive
73	Male	CHX	10 months	A	positive	na	positive
74	Male	CHX	1 year	A	positive	Spoligo type9	positive
75	Male	SMX	1 year 1 month	L	positive	na	positive
76	Female	CHX	1 year	A	positive	na	positive
77	Female	CHX	1 year	L & A	positive	Spoligo type9	positive
78	Female	CHX	2 years	L	positive	Spoligo type9	positive
79	Male	CHX	1 year	L	positive	Spoligo type9	positive
80	Male	CHX	1 year	L & A	positive	Spoligo type9	positive
81	Male	CHX	1 year	L	positive	Spoligo type9	positive
82	Male	CHX	2 years	L	positive	Spoligo type9	positive
83	Female	CHX	1 year	A & L	positive	Spoligo type9	positive
84	Female	LIMX	3 years	L	positive	Spoligo type9	positive
85	Female	CHX	2 years 11 months	A	positive	Spoligo type9	positive

86	Male	CHX	2 years 11 months	L	positive	Spoligo type9	positive
87	Female	HEX	3 years 2 months	A & L	positive	Spoligo type9	positive
88	Female	HEX	6 years 3 months	L	positive	Spoligo type9	positive
89	Female	HEX	5 years	L	positive	Spoligo type9	positive
90	Female	HEX	4 years 5 months	A	positive	Spoligo type9	positive
91	Female	HEX	10 years 5 months	L	positive	Spoligo type9	positive
92	Female	HEX	4 years 7 months	A	positive	Spoligo type9	positive
93	Female	HEX	11 years	L	positive	Spoligo type9	positive
94	Female	HEX	6 years	A	positive	Spoligo type9	positive
95	Female	HEX	6 years 6 months	L	positive	na	positive
96	Female	HF	4 years 6 months	A	positive	Spoligo type9	positive
97	Female	HF	2 years 7 months	L	positive	Spoligo type9	positive
98	Female	HF	1 year 8 months	A	positive	Spoligo type9	positive
99	Female	HF	2 years 1 month	L&A	positive	Spoligo type9	positive
100	Female	HF	2 years 10 months	A	positive	Spoligo type9	positive
101	Female	BF	4 years 4 months	A	positive	Spoligo type11	positive
102	Female	HF	3 years	A	positive	Spoligo type9	positive
103	Female	HF	2 years 10 months	H	positive	Spoligo type9	positive
104	Female	HF	3 years	A	positive	Spoligo type9	positive
105	Female	HF	2 years 11 months	A	positive	Spoligo type9	positive
106	Female	HE	2 years 10 months	A	positive	Spoligo type9	positive
107	Female	HE	1 year 6 months	A	positive	Spoligo type9	positive
108	Male	HEX	1 year	H	positive	na	positive
109	Male	HEX	1 year	H	positive	na	positive
110	Male	HEX	1 year	H	positive	na	positive
111	Female	AAX	2 years	H	positive	na	positive
112	Female	HFX	2 years	H	positive	na	positive
113	Male	AAX	2 years	H	positive	na	positive
114	Male	AAX	2 years	H	positive	na	positive
115	Female	AAX	2 years	H	positive	na	positive
116	Male	BRBX	2 years	H	positive	na	positive
117	Female	HFX	2 years	H	positive	na	positive
118	Female	HF	2 years	H	positive	na	positive
119	Male	AAX	1 year	H	positive	na	positive
120	Female	HF	7 years	H	positive	na	positive
121	Female	BRBX	7 months	H	positive	na	positive
122	Female	HF	7 months	H	positive	na	positive
123	Female	HF	1 year	H	positive	na	positive
124	Female	HF	1 year	H	positive	na	positive
125	Male	BRBX	5 months	L	positive	na	positive
126	Female	HEX	1 year	H	positive	na	positive
127	Female	HEX	1 year	H	positive	na	positive

Samples were obtained from 127 cattle (73 females; 49 males, 5 unknown) confirmed to be naturally M. bovis-infected using the single intradermal comparative cervical tuberculin test (SICCT) and the laboratorial culture. The cattle breeds and codes used in table are available at https://www.gov.uk/guidance/official-cattle-

breeds-and-codes. Sera of 1-107 animals of this cohort were collected at an abattoir, and for each animal the laboratorial culture results of a lymph node pool were positive for M. bovis. Sera of 108-127 animals in this cohort, individual lymph nodes plus a lung biopsy were cultured, and a positive M. bovis culture result was obtained for each animal from at least one node. na, not applicable.

Supplementary Table S2: Characteristics of SICCT-negative cattle cohort

Sample No.	Breed code	Gender	SICCT
1	MOX	Female	Negative
2	BFX	Female	Negative
3	MO	Female	Negative
4	AYX	Female	Negative
5	BFX	Female	Negative
6	HFX	Female	Negative
7	AYX	Female	Negative
8	MOX	Female	Negative
9	AYX	Female	Negative
10	MOX	Female	Negative
11	AYX	Female	Negative
12	HFX	Female	Negative
13	SMX	Female	Negative
14	MOX	Female	Negative
15	PAX	Female	Negative
16	MOX	Female	Negative
17	HFX	Female	Negative
18	AYX	Female	Negative
19	AYX	Female	Negative
20	AYX	Female	Negative
21	AYX	Female	Negative
22	MOX	Female	Negative
23	HFX	Female	Negative
24	BFX	Female	Negative
25	MOX	Female	Negative
26	BFX	Female	Negative
27	BF	Female	Negative
28	BFX	Female	Negative
29	AYX	Female	Negative
30	MOX	Female	Negative
31	MOX	Female	Negative
32	AYX	Female	Negative
33	BFX	Female	Negative
34	HFX	Female	Negative
35	HFX	Female	Negative
36	HO	Female	Negative
37	MOX	Female	Negative
38	HFX	Female	Negative
39	AYX	Female	Negative
40	BFX	Female	Negative
41	MOX	Female	Negative
42	BFX	Female	Negative
43	MOX	Female	Negative
44	BFX	Female	Negative
45	AY	Female	Negative
46	JE	Female	Negative

47	BF	Female	Negative
48	AYX	Female	Negative
49	MO	Female	Negative
50	BF	Female	Negative
51	HFX	Female	Negative
52	MOX	Female	Negative
53	MOX	Female	Negative
54	BF	Female	Negative
55	AYX	Female	Negative
56	BFX	Female	Negative
57	BF	Female	Negative
58	BFX	Female	Negative
59	BF	Female	Negative
60	MOX	Female	Negative
61	BFX	Female	Negative
62	AYX	Female	Negative
63	BF	Female	Negative
64	BF	Female	Negative
65	BFX	Female	Negative
66	AYX	Female	Negative
67	BFX	Female	Negative
68	BFX	Female	Negative
69	AYX	Female	Negative
70	BFX	Female	Negative
71	BFX	Female	Negative
72	BFX	Female	Negative
73	HFX	Female	Negative
74	AYX	Female	Negative
75	BFX	Female	Negative
76	MOX	Female	Negative
77	MOX	Female	Negative
78	MOX	Female	Negative
79	SMX	Female	Negative
80	MOX	Female	Negative

Samples were obtained from 80 female cattle confirmed to be negative using the SICCT. The cattle breeds and codes used in table are available at https://www.gov.uk/guidance/official-cattle-breeds-and-codes.

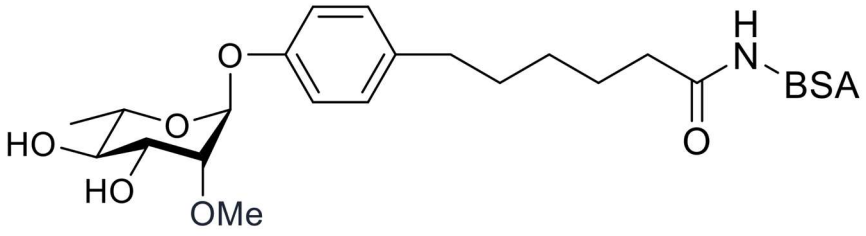

Figure S1. The structure of synthetic M. bovis PGL monosaccharide. *Approximate average glycan loadings (based on MALDI analysis) were 39 monosaccharides per BSA molecule [1-3].*

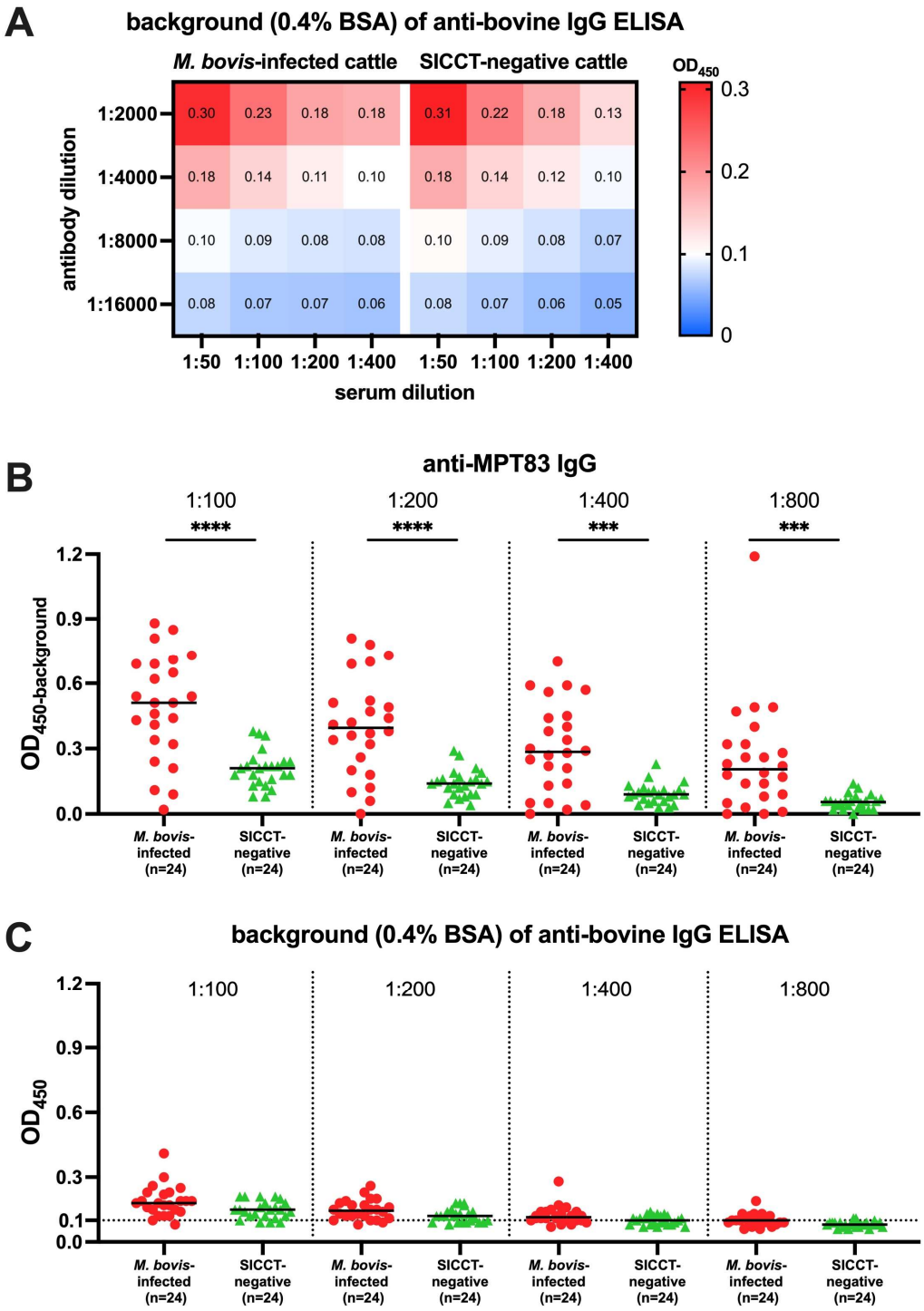

Figure S2. Anti-bovine IgG background and anti-MPB83 IgG signals in ELISAs for different serum dilutions. *Heatmap depicting background (0.4% BSA coated) OD values of one random sample in M. bovis-infected and SICCT-negative cattle, respectively (#15 in Table S1 and #15 in Table S2), measured by ELISA at different detection antibody (1:2000 - 1:16000) and serum dilutions (1:50 - 1:400) (**A**). OD values for background and anti-MPB83 IgG (500 ng/well coated) in 24 randomly selected serum sample from M. bovis-infected (#24-47 in Table S1) and SICCT-negative (#21-44 in Table S2) cattle, respectively, were measured by ELISA of different serum dilutions (1:100 - 1:800). The anti-bovine IgG antibody was*

*diluted 1:8000. ELISA results of background values are displayed as optical densities at 450 nm ($OD_{450;}$ y-axis in **C**); anti-MPB83 IgG levels are displayed as optical density at 450 nm corrected for background OD values ($OD_{450\text{-background}}$; y-axis in **B**). The median values of each group are indicated by horizontal lines; difference between antibody levels in sera were determined by Mann-Whitney U-tests; P-values: ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001. inf., infected; neg., negative (**B**, **C**).*

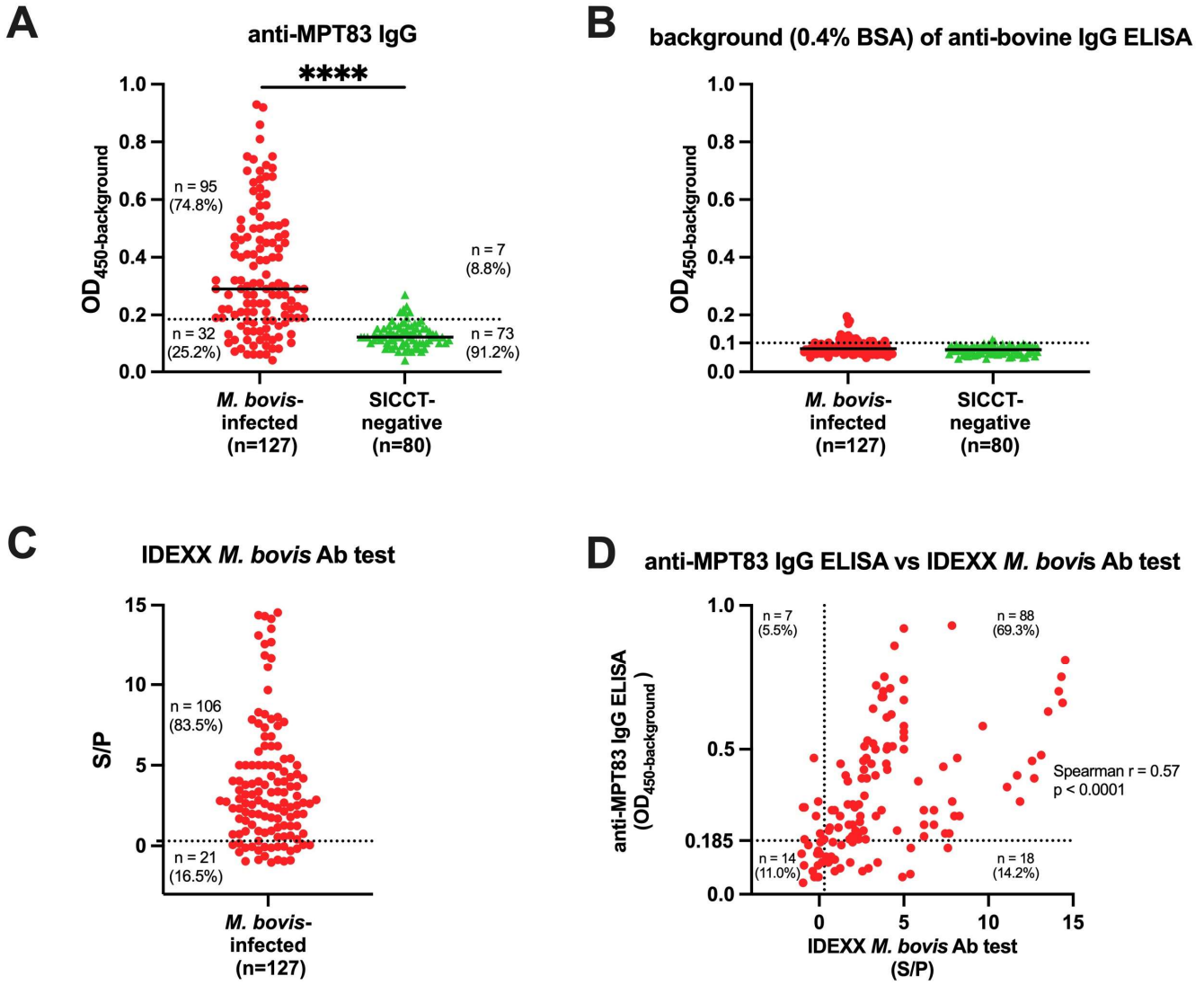

Figure S3. Anti-MPT83 IgG levels in serum samples from naturally M. bovis-infected and SICCT-negative cattle. *Anti-MPT83 IgG (**A**) and background (0.4% BSA, **B**) levels were measured by ELISA in sera from M. bovis-infected (n=127, red dots) and SICCT-negative cattle (n=80, green triangles). ELISA results are displayed as optical density at 450 nm corrected for background OD values ($OD_{450\text{-}background}$; y-axis). The cut-off value for anti-M. bovis PGL antibodies were determined by Youden's index ($OD_{450\text{-}background}$ >0.185, indicated by dashed line in **A**). Difference between antibody levels in sera were determined by Mann-Whitney U-tests; P-values: ****p ≤ 0.0001. IDEXX M. bovis Ab test results (**C**) are presented as sample-to-positive ratios (S/P) derived by subtracting the mean kit negative-control optical density (OD) from each sample and dividing this value by the corrected positive-control value (mean positive-control OD minus mean negative-control OD). The S/P ≥ 0.3 (dashed line in **C**) is set as cut-off value according to the kit instruction. The correlation between anti-MPT83 IgG ELISA and IDEXX M. bovis Ab test is shown in panel **D**.*

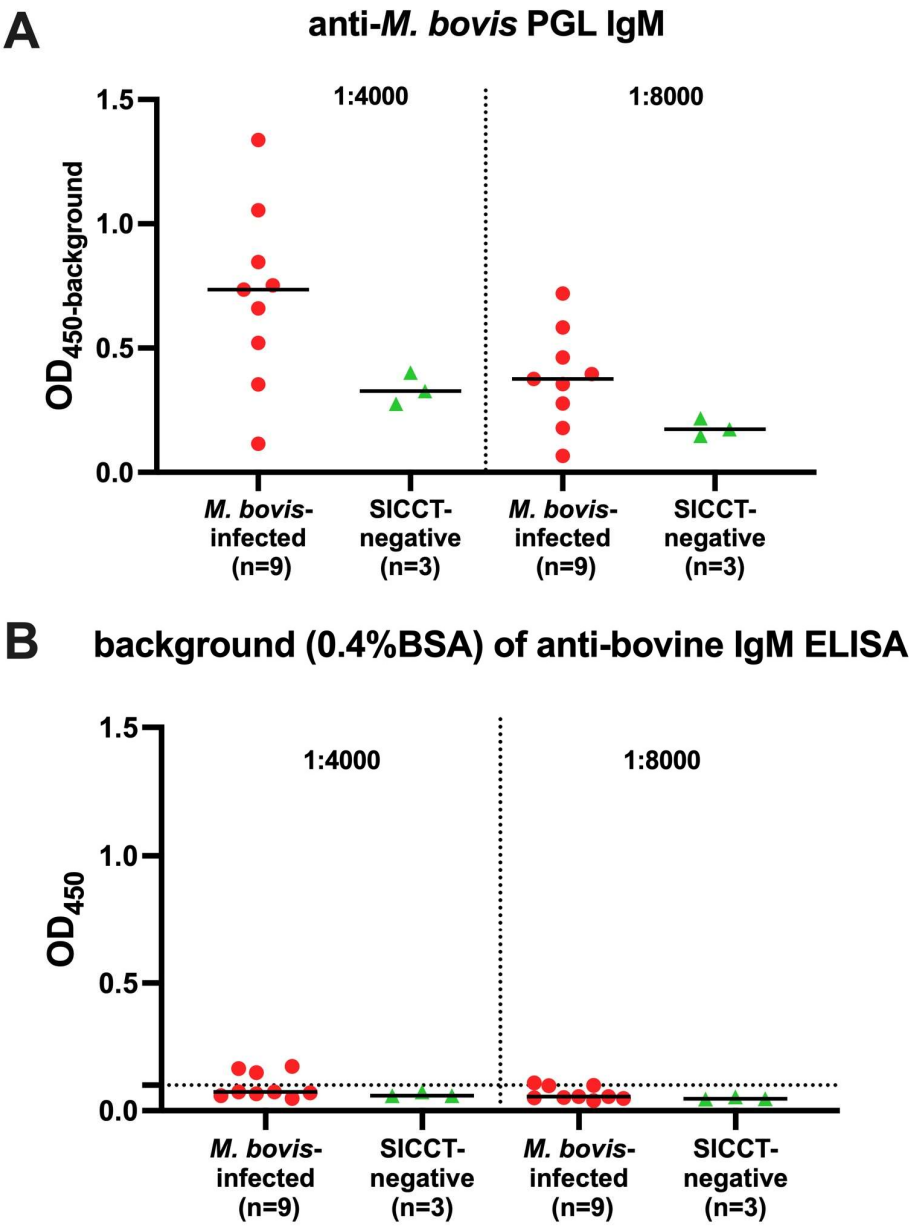

Figure S4. Anti-M. bovis PGL IgM of different anti-bovine IgM antibody dilutions. *Nine anti-MPT83 IgG positive samples in M. bovis-infected (#15-23 in Table S1, red dots) and three SICCT-negative cattle (#15-18 in Table S2, green triangles) were tested in anti-M. bovis PGL ELISAs. M. bovis PGL (200 ng/well,* ***A****) and 0.4% BSA (****B****) were coated in 96-well microplates. Serum samples were diluted 1:400, and anti-bovine IgM antibody was diluted 1:4000 and 1:8000. Background values are displayed as optical densities at 450 nm (OD_{450}); results of antibody levels are displayed as optical density at 450 nm corrected for background OD values ($OD_{450\text{-background}}$). The median values of each group are indicated by horizontal lines.*

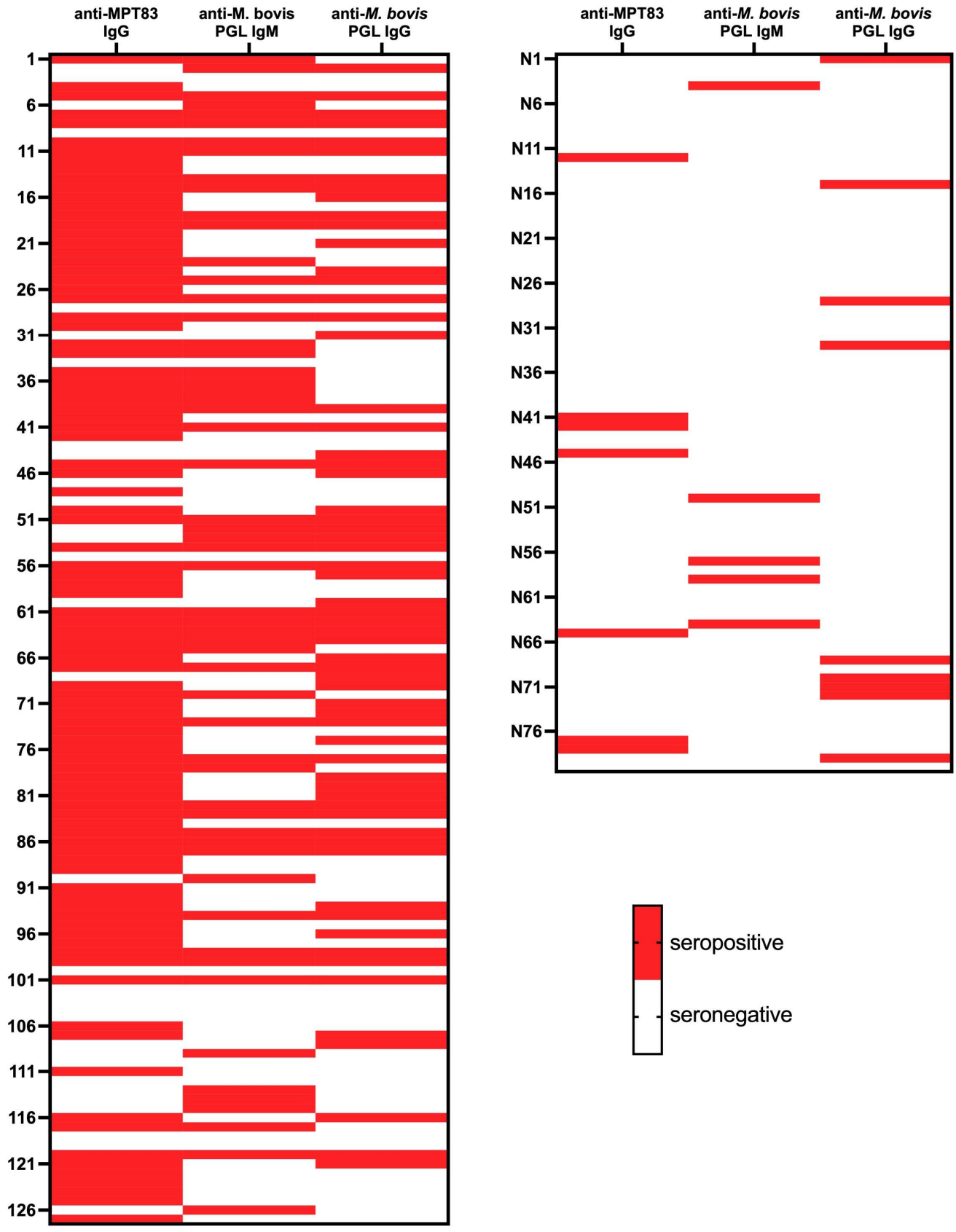

Figure S5. Heatmap of ELISA results. *Anti-M. bovis PGL IgM and anti-MPT83 IgG ELISA results in cohorts A (n=127, left panel) and B (n=80, right panel) using cut-off values shown in* ***Fig. S3****,* ***Fig. 2*** *and* ***Tab. 1****; values above the cut-off for each biomarker are considered seropositive (marked in red).*

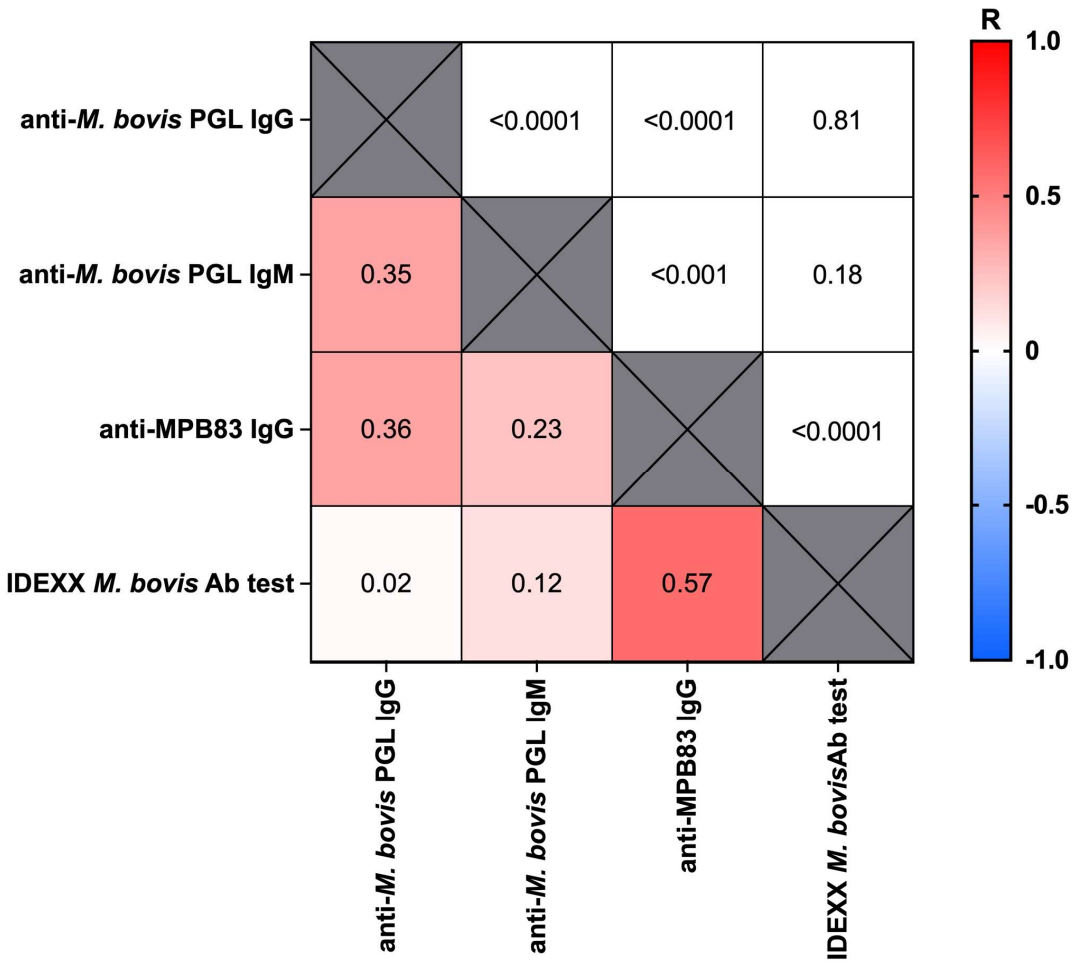

Figure S6: Correlation between antibody levels. *Serum samples of M. bovis infected cattle (n=127) were assessed by anti-M. bovis PGL IgM/-IgG ELISA, anti-MPB83 IgG by ELISA, and IDEXX M. bovis Ab test. R values, the Spearman correlation coefficients, are depicted in bottom left corner of the heatmap. The corresponding p-values, indicating the significance level of the observed correlation between the different tests, are shown in upper right corner in white cells.*

Reference:

1. van Dijk, J.H.M., G.A. van der Marel, and J.D.C. Codée, *Developments in the Synthesis of Mycobacterial Phenolic Glycolipids.* Chem Rec, 2021. **21**(11): p. 3295-3312.
2. Barroso, S., et al., *Total Synthesis of the Triglycosyl Phenolic Glycolipid PGL-tb1 from Mycobacterium tuberculosis.* Angewandte Chemie International Edition, 2012. **51**(47): p. 11774-11777.
3. Barroso, S., et al., *Total Synthesis of the Phenolic Glycolipid Mycoside B and the Glycosylated p-Hydroxybenzoic Acid Methyl Ester HBAD-I, Virulence Markers of Mycobacterium tuberculosis.* European Journal of Organic Chemistry, 2013. **2013**(21): p. 4642-4654.