Veterinary Laboratories Agency

Specificity Trial of the BOVIGAM® IFN-Gamma Test in GB Cattle

Dr Martin Vordermeier and Dr Katie Ewer

TB Research Group, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB

Funded by Defra under surveillance project SB4021

April 2006

Contents:

1. **Background to trial and the BOVIGAM® test**
2. **Objectives, study design, scientific limitations**
3. **Methodology**
4. **Results**
5. **Discussion**
6. **Recommendations**
7. **References**
8. **Appendices**

Executive summary:

- **A trial to determine the specificity of the BOVIGAM® IFN-γ test detecting bovine tuberculosis in cattle was performed using animals from GB herds**
- **Test antigens were: avian and bovine tuberculin PPD, and a cocktail of peptides from the *Mycobacterium bovis* antigens ESAT-6 and CFP-10**
- **1274 animals from 24 farms were sampled (21 from Animal Health Office, Reading and 3 from AHO Leicester regions)**
- **Results from 1102 samples could be used for evaluation and the following specificity levels were determined:**

  PPD-B minus PPD-A: 96.3 % (95% CI 95.3-97.4%)
  ESAT-6/CFP-10 peptides: 95.8 % (95% CI 94.7-97.0%)
  PPD-B minus PPD-A AND ESAT-6/CFP10 peptides combined: 98.9 % (95% CI 98.4-99.6%)

- **When results from herds that had imported animals from herds with a recent history of bovine TB were removed, specificity levels were re-calculated to be on the basis of 874 animals:**

  PPD-B minus PPD-A: 96.7 % (95%CI 95.6-97.8%)
  ESAT-6/CFP-10 peptides: 97.0 % (95% CI 95.9-98.1%)
  PPD-B minus PPD-A AND ESAT-6/CFP10 peptides combined: 99.2 % (98.6-99.8%)

- **The results of this trial are therefore in line with previously published data**
- **Results have been discussed and a list of recommendations in respect to test interpretation, further studies, and test implementation has been made.**

1.Background

1.1 Policy rationale

Defra has a statutory duty to control tuberculosis in farm animals in Great Britain under the Animal Health Act of 1981, the Tuberculosis Orders, the Zoonoses Orders and various EU Directives. Using a diagnostic test for bovine tuberculosis (BTB) that may have increased sensitivity for detecting BTB when used in conjunction with the currently used comparative tuberculin skin test could help reduce the rate of increase of BTB in GB, reduce the length of time herds are under movement restriction and, thus, improve BTB control.

Any cost benefit analysis of using the IFN-γ test will require knowledge of the baseline specificity of the test under GB conditions.

1.2. The BOVIGAM® IFN-γ blood test

The IFN-γ test is an *in vitro* laboratory-based immuno-diagnostic test detecting gamma interferon (IFN-γ) that is produced after the stimulation of blood cells with stimuli such as bovine tuberculin. The test is performed in two stages. Firstly, blood samples are transported to the laboratory within 24 h of sampling, and cultured at 37°C in the presence of bovine or avian tuberculin or a cocktail of synthetic peptides from the mycobacterial proteins ESAT-6 and CFP-10. After 24 h incubation, plasma supernatants are collected, and the amount of IFN-γ produced quantified by enzyme-immuno-assay (EIA) using the commercially available BOVIGAM® kit. A test result is interpreted as positive for bovine tuberculosis when the blood cells from an infected cow produce more IFN-γ after stimulation with bovine tuberculin than after stimulation with avian tuberculin. Practical advantages of this test include (36): (i) flexibility in its interpretation by setting appropriate cut-offs; (ii) not requiring a repeat farm visit to read the test; (iii) no interference with the host's immune status, thus assays can be repeated more frequently than tuberculin skin tests; (iv) it is highly amenable to the inclusion of defined antigens that allow the differentiation between infection and vaccination (*differential diagnosis*, see below). It is now also accepted that the BOVIGAM® test detects animals that escape skin testing, probably because it detects animals earlier after infection than tuberculin skin testing (e.g. 11, 17, 20, 21).

Table A1 (appendix 1) gives reported sensitivity and specificity ranges of the BOVIGAM® test using bovine and avian PPD, based on data from 19 published studies performed in 11 countries/regions (1, 4-6, 8, 11, 13, 15, 16, 18, 24, 28-30, 32, 34-36). The BOVIGAM® test is generally considered to be more sensitive than tuberculin skin testing (36), whilst specificities

have been reported that, although not widely dissimilar to those seen with tuberculin skin testing, were lower in most studies than those reported for SICCT. However, direct comparisons of the two tests in the same study have rarely been made.

The majority of infected animals will be positive in both assays, but proportions of animals will be IFN-γ-positive/skin test negative, IFN-γ-negative/skin test positive (e.g. 4, 20, 24, 28, 30, 32, 34, 36), or negative in both tests (e.g. 34, 36). Therefore, the most beneficial application of this test is alongside the tuberculin skin test as a ***parallel test*** in herds with high disease prevalence or persistent infection that cannot be cleared with the tuberculin skin test. This will increase the overall diagnostic sensitivity to detect infected animals and will thus have a major impact on disease control. Table A2 (appendix) presents published and unpublished data illustrating this point. Please also refer to a comprehensive review of this subject that was recently published (9). Further information and a conceptual discussion of the infection biology of tuberculosis with relevance to the interpretation of results from tests probing cellular immunity like tuberculin skin test and the BOVIGAM® IFN-γ assay have been attached as appendix 2.

2. Objectives and deliverables

2.1. Aims and Objectives

- To determine the specificity of the BOVIGAM® IFN-γ test using avian and bovine PPD or a peptide cocktail of ESAT-6 and CFP-10 (30) in GB.
- To optimise test protocols (SOP) and procedures including training and quality control approaches.

2.2. Specific deliverables

- Estimate of specificity of IFN-γ test using both tuberculin and specific antigens in the absence of previously applied tuberculin skin testing.
- Estimate of reproducibility of test by comparison of results obtained at VLA Luddington and VLA Weybridge on a proportion of samples tested.

2.3. Scientific dependencies

- As, at the time of this report, no test-positive animals had been purchased and examined by post-mortem and culture, no additional data was available on true infection status of test-positive animals, and therefore on true specificity (see: *recommendations* section).
- A tuberculin skin test may boost subsequent IFN-γ responses. However, we were restricted to pre-skin test sampling and, therefore, could not examine this possibility. Fortunately, subsequent studies in another project (SE3033) indicated that the comparative tuberculin skin test as used, in contrast to the caudal fold test, does not boost subsequent IFN-γ responses (see: *discussion* section).

3. Methodology

Reading Animal Health Office (and during the later stage of the trial, Leicester AHO) randomly selected herds due for routine tuberculin skin testing. Blood samples (7 ml heparinized blood in one vacutainer/animal) were taken on the day of applying the tuberculin skin test and blood sampling for brucellosis surveillance, packaged in temperature-stable packaging material (Saf-T-Paks) and dispatched to VLA Luddington and in some cases to VLA Weybridge. Target: 1000-1500 animals. 300 samples were tested both at VLA Weybridge and VLA Luddington to provide quality control data.

Details:

- Gamma Interferon testing (BOVIGAM®, Prionics) was performed on bloods taken at the time of a Brucella blood sampling visit using duplicate incubations with the following antigens: *nil*, *avian tuberculin, bovine tuberculin, ESAT-6/CFP-10 peptide cocktail, positive control (either SEB).* Interpretation criteria for positivity (30): OD450 with PPD-B minus OD450 with PPD-A > 0.1, or OD450 with peptide cocktail minus OD450 with medium control > 0.1.
- Herd selection criteria: In order to calibrate the γ-IFN test and assess its specificity it was necessary to undertake this trial in areas of low TB prevalence. For this reason the Reading and Leicester AHOs were chosen so that they could select herds in 4-yearly testing areas, with no history of TB test reactors or slaughterhouse cases in the last 8 years. These herds were also required to be at least 10 km from the nearest TB incident disclosed in the last 5 years.
- Blood sensitisation was initiated on the day following blood sampling. IFN-γ testing was performed as specified in VLA Standard Operating Procedures in operation at the time this trial was conducted.

4. Results

4.1 Recruitment

Samples were tested from 1234 animals derived from 24 farms; 21 from Reading AHO and 3 from Leicester AHO. 284 samples failed QC criteria and were retested where possible; 132 retests gave valid results, with 75 failing QC criteria a second time and 57 having insufficient sample for retesting.

4.2 Specificity estimates

A total of 1102 valid results were obtained, of which 79 (7.2%) were positive by current criteria, either to tuberculin (bovine tuberculin [PPDB]–avian tuberculin [PPDA), ESAT6/CFP10 peptide cocktail, or both. Results to each antigen by farm are shown in Table 1.

Farm Name	No. Sampled	No. Results	Tuberculin B-A Positive (%)*	ESAT6 /CFP10 Peptide cocktail Positive (%)*	Both Positive (%)*
1	45	37	0	4 (10.8)	0
2	54	53	1 (1.9)	3 (5.7)	3 (5.7)
3	22	18	1 (5.6)	0	1 (5.6)
4	29	25	0	0	1 (4.0)
5	57	55	4 (7.3)	5 (9.1)	2 (3.6)
6	81	79	1 (1.3)	0	1 (1.3)
7	98	88	1(1.1)	9 (10.2)	1 (1.1)
8	98	93	1 (1.1)	1 (1.1)	1 (1.1)
9	100	95	3 (3.2)	2 (2.1)	1 (1.1)
10	15	15	2(13.3)	0	0
11	35	34	4 (11.8)	0	0
12	54	52	4 (7.7)	0	0
13	42	41	2 (4.9)	1 (2.4)	0
14	94	93	4 (4.3)	0	0
15	25	25	1 (4.0)	0	0
16	39	35	1 (2.9)	0	0
17	83	80	1 (1.3)	1 (1.3)	0
18	50	47	0	11 (23.4)	0
19	28	26	0	0	0
20	9	5	0	0	0
21	41	41	0	0	0
22	90	22	0	0	0
23	12	10	0	0	0
24	33	33	0	0	0
Total	1234	1102	31	37	11

Table 1. Results from individual herds.

Specificity estimates and 95% confidence intervals (95% CI) are summarised in Table 2. **Specificity was 96.3% for tuberculin, 95.8 for the peptide cocktail and 98.9% where a positive response to both antigens was obtained.** This data is in line with published data from other studies; a recent review of published studies (9) showed the median specificity for the BOVIGAM® assay using PPD to be 96.6% (range 87.7-99.2%). Our results are therefore in line with published data. Skin-testing performed at the time of blood collection revealed 5 inconclusive reactors on 4 farms; all were resolved on retesting.

Using eartag numbers, movement histories of all 79 animals testing positive were searched for in the Cattle Traceability System database. Matches were found for 65 (82.2%) animals. Four of these, from four separate locations, had a history of residence on a farm with at least one previous TB incident. As these animals had not had their disease status established by post mortem examination at the time of preparing this report, these farms were removed from the dataset as they could not be definitively identified as 'TB free' given the presence of suspect animals in the herd. On one other farm with a high frequency of response to the peptide cocktail, unidentified disease had caused several unexplained deaths and this herd was also removed leaving a subset of 874 animals. Specificity estimates and 95% CI are shown for both datasets in Table 2. **Specificity for the 874 animal dataset was 96.7% for tuberculin, 97.0 for the peptide cocktail and 99.2 % where a positive response to both antigens was obtained.**

	Entire dataset (n=1102)			Subset (n=874)		
Result	n positive (%)	Specificity	95% CI *	n positive (%)	Specificity	95% CI *
B-A	42 (3.8)	96.3	95.3 – 97.4	23 (2.6)	96.7	95.6 – 97.8
ESAT6 / CFP10 peptide cocktail	46 (4.2)	95.8	94.7 – 97.0	20 (2.3)	97.0	95.9 – 98.1
Either positive	79 (7.2)	93.3	92.0 – 94.7	50 (5.7)	94.6	93.2 – 96.0
Both positive	11 (1.0)	98.9	98.4 – 99.6	7 (0.8)	99.2	98.6 – 99.8

Table 2. Specificity estimates and confidence intervals for the IFNγ test for bovine tuberculosis. (***CI = confidence intervals)**

4.3 Quality Control

A total of 314 samples were submitted to VLA Weybridge for parallel testing with identical samples submitted to Luddington. Results were obtained for 285 samples as 29 were refrigerated overnight and were therefore unsuitable for processing. For tuberculin B-A, agreement between laboratories was 96.1%, while for the peptide cocktail 95.1% of samples gave the same result at both sites.

5. Discussion

5.1. The results of this specificity trial are in agreement with a vast array of previously published data from a number of different countries (rev. in (9)), with the levels defined in this study falling within the top half of all published earlier results (9). Interestingly, the specificity results for the comparative analysis of tuberculin responses were virtually identical to those reported in an earlier study with a small number of GB cattle, conducted by Vordermeier (96.6 % compared to 96.3 –96.7 % in the present study, Vordermeier, report to project SE3005), thereby confirming and extending these earlier GB data.

5.2. Sensitivity and specificity of a test are not isolated values. Indeed, they are linked and dependent on each other in respect to the cut-offs applied. As we did not include in this 'specificity trial' animals with confirmed bovine tuberculosis, which in conjunction with the data from truly negative animals, would have allowed us to determine the relative specificity and sensitivity of the IFN-γ in respect to different cut-offs, we had to use cut-offs determined in earlier studies. Although this was vindicated by the results, as we obtained identical specificity values in the present larger study than in the previous one (See report to project SE3005), it would be of value to re-assess cut-offs in respect to different specificity and sensitivity levels by using data from confirmed bovine TB cases in conjunction with the data from negative animals. We, therefore, recommend including data from culture-confirmed animals obtained in the national IFN-γ field trial and in *ad hoc* usage so that Receiver Operator Curve (ROC) analysis can be performed.

5.3. During our analysis it became evident that some of the herds taking part in this study contained animals that were imported from herds with a recent history of bovine TB. Interestingly, such imported animals tested positive after applying the BOVIGAM® assay. However, at the time of this report we had not purchased and examined these animals by

post-mortem and culture. Therefore, we excluded these herds from the secondary analysis as their infection status remained undefined.

5.4. ESAT-6/CFP-10 positive/tuberculin-negative animals.

One herd contained a relatively high number of cattle that tested positive only when ESAT-6/CFP-10 peptides were applied, but were negative when avian and bovine PPD responses were compared. Similar animals were found, albeit at a much lesser percentage, in two additional herds (see table 1). Whilst such animals did not pose a problem in the remaining 21 herds tested, such cases were clustered in these three herds. This should be investigated further. We hypothesize that these animals were exposed to non-TB mycobacterial species that express ESAT-6 and CFP-10 (e.g. *M. kansasii* and *M. szelgai,* (2)). These species, in particular *M. kansasii,* have been isolated from GB cattle (Keith Jahans, VLA Weybridge, personal communication). We have shown, on an individual epitope basis, complete cross-reactivity between *M. bovis* and *M. kansasii* ESAT-6 and CFP-10 (Vordermeier, Arend, Ottenhoff, unpublished data). In addition, Waters and co-workers, after infecting cattle with *M. kansasii*, could also demonstrate that *M. bovis* ESAT-6 and CFP-10 were recognized in such animals in the BOVIGAM® assay, further demonstrating the cross-reactivity between these *M. kansasii* and *M. bovis* proteins. However, when they applied avian and bovine PPD, they found that the *M. kansasii* infected cattle tested negative in the BOVIGAM® test (Waters *et al.*, presentation at *M. bovis* IV conference, Dublin, August 2005; and Ray Waters, personal communications). Waters *et al.*'s results are therefore similar to the ones observed in the specificity trial in particular herds, and consequently support our hypothesis. We recommend, until further investigation has led to a classification of such 'ESAT-6/CFP-10-positive only' cattle, that these animals should not be classified as positive for bovine TB on the basis of a positive IFN- response to ESAT-6 and CFP-10 only, and that the comparison between PPD-B and PPD-A should be used to classify animals in such herds. As such animals are concentrated to particular herds, they do not pose a general problem as these herds could still be assessed using PPD only. However, such herds should be monitored and their locations plotted to determine whether such herds occur in particular geographic clusters. Attempts should also be made to purchase some of these animals to perform post-mortem examinations and to attempt culture of mycobacterial species different from *M. bovis.*

5.5. Time interval between blood sampling and test initiation.

The blood stimulation was initiated in this trial on the day after blood sampling. The reasons for this were firstly logistical as it was not possible to transport blood samples from the sites of sampling to the testing laboratory before 19:00 h on the sampling day. Secondly (see below), our data from previous studies suggest that blood storage overnight before setting up the blood cultures significantly increases test specificity.

In practical terms the issue relates to whether blood should be tested on the day of sampling (i.e. within 8 h), or could be left overnight (i.e. within 24 h). Previous investigations have all used PPD-A and PPD-B as test antigens. Studies in New Zealand and the USA have concluded that about equal portions of animals could be defined as positive when the BOVIGAM® test was performed on the day of sampling or within 24 h of sampling (28, 33). This was despite the observation that the OD values after overnight storage of the blood samples were substantially lower than when the blood culture was started on the day of sampling. These results were obtained with reactor animals with confirmed TB, see also Figure A1 in appendix 1, and with experimentally sensitised steers, and formed the basis of NZ and USA policy of starting the assay on the day following sampling (within 24 h).

Our own results (Vordermeier, unpublished, Vordermeier, final project report to Defra project SE3005, and (31)) confirmed these findings in that we could also demonstrate that, despite decreases in signal strength (OD450), we did not observe a reduction in the numbers of experimentally infected cattle detected (31), or a modest drop in sensitivity when we assessed SICCT field reactors with confirmed TB (Vordermeier, final report SE3005, also Figure A1 in appendix 1) when we applied a high cut-off value (PPD-B minus PPD-A > 0.1 OD450). This loss in sensitivity after overnight storage could be compensated by using a lower cut-off: PPD-B minus PPD-A >0.05 OD450, Fig. 1). **Most dramatically however, we observed an increase in specificity when the blood was kept overnight rather than cultured freshly (Fig. A1, from about 84 to 97%, and from around 80% to 88%, for 0.1 and 0.05 OD450 cut-off values, respectively).** Incidentally, the specificity levels determined in the present, larger study were identical to those found in this earlier study, and these earlier results are therefore relevant to the present study. Such an increase in specificity following overnight storage has also been observed by Ryan et al. (28) (see also Fig. A1 in appendix 1).

In contrast to these findings that suggest that overnight storage of blood has no significant impact on sensitivity, a recent paper by Gormley et al. (14) suggests that the delay in blood

culture reduces test sensitivity significantly in some animal groups. In this paper three groups of IFN-γ test-positive animals were assessed, namely, 43 SICCT-reactors with visible lesions (confirmed TB), a group of SICCT-positive animals from surveillance operations (only 6/29 had confirmed TB), and a group of 60 SICCT-negative animals that were IFN-γ positive (no post-mortems were performed). Applying the BOVIGAM® test to group 1 animals within 8 or 24 h did not decrease sensitivity significantly (42/43 still positive when tested within 24 h of sampling) in line with what the other studies described above had also shown. However, when testing the other two groups, Gormley et al. (14) showed a dramatic decrease in the percentage of animals testing positive after 24 h storage compared to 8 h. This was particularly the case for the SICCT-negative animals (reduction of test positivity from 100 to about 52 %). However, it is impossible to assess the disease status of this skin-test negative group (i.e. were they infected at the time of testing or not) as no post mortem examinations were conducted. It is therefore also impossible to assess if this decrease in the number of animals testing BOVIGAM®-positive is due to a decrease in sensitivity due to overnight storage, or indeed an increase in specificity as we have seen, due to the detection of less false-positive animals. The same proviso has to be applied to some degree to group 2 animals (only 21 % of animals presented as VL). Unfortunately, truly negative animals were not assessed in this study.

Our recommendation, based on both scientific arguments of increased specificity and the logistical and financial constraints imposed by starting the test on the day of sampling, therefore remains unchanged, i.e. blood cultures should be initiated on the day following blood sampling. However, one should keep in mind that the use of this assay can be tailored to particular situations, i.e. if extremely high sensitivity is required, the test could be performed on the day of sampling, or results of tests initiated on the day after sampling could be interpreted using different (lower) cut-offs. However, this would certainly also result in decreased specificity.

5.6. Blood sampling in relation to skin test application.

Early data by Rothel et al. (27) suggested that IFN-γ responses were affected by the application of tuberculin skin tests: They observed increases in the OD450 values after *in vitro* stimulation with avian and bovine PPD between 7 and 59 days post-skin test. The test applied was the caudal fold test (CFT). Since then numerous studies have looked at the development of IFN-γ responses post-skin test, and reached conflicting conclusions. However, by comparing these studies, some general conclusions can be drawn.

Ryan et al. (28) evaluated the BOVIGAM® test 8-28 days post CFT, and concluded that taking blood during this time interval increased test sensitivity. These results now form the basis of current NZ BOVIGAM® test policy. Whipple et al. (33) confirmed that the CFT boosted BOVIGAM® responses by day 3 post-CFT for a period of 63 days post-CFT, but applying the SICCT at day 63 post-CFT did not result in an additional IFN-γ response boost. They furthermore demonstrated that higher signal strengths could be obtained with blood taken 3 days post-CFT and stored overnight compared to blood sampled at the same time but tested immediately (within 2 h). These results have been used to obtain USDA approval to use the BOVIGAM® test 3 days post-CFT. These results were confirmed and extended in a recent paper by Palmer et al. from the same group (23): IFN-γ responses were boosted for about 1 week after CFT application, but not after an SICCT. We have also shown that application of the SICCT does not significantly boost IFN-γ responses (Fig. A2 in appendix 1, (31), and Coad, Whelan, and Vordermeier, submitted) over a period of 38 days post-SICCT (Figure A2, upper panel), as has Gormley et al. for up to 60 days post-SICCT (14). Doherty et al., (10) also showed that application of the SICCT did not boost IFN-γ responses measured 7 days later.

Furthermore, our data, as well as that of Gormley et al (14), demonstrated that blood could be taken 3 days post-SICCT application without affecting test sensitivity (Figure A2, appendix 1). Interestingly, Gormley's data showed that the decrease in responses between blood stored for 8 h or 24 h is less pronounced at 3 days post-skin test than at the day of SICCT injection or 10 days post-SICCT. Our data supports this observation (Fig. A2). In addition, the test interpretation based on the OD450 differential between PPD-B and PPD-A did not indicate a drop of sensitivity between blood tested on day of testing or overnight storage and sampling on days 0, 3, and 10 post skin test application. So, in conclusion, the specificity results

obtained in the present trial, which were obtained with pre-SICCT blood, are relevant also to post-SICCT blood taken within 3 days of tuberculin injection.

6. Recommendations

1. Further evaluation of data obtained from this trial.

1.1 As we have not yet determined the infection and disease status of the IFN-γ positive animals by post mortem examination and *M. bovis* culture, we removed from our secondary analysis results from herds that had imported animals from herds with a history of confirmed bovine TB. However, we recommend that these herds should be re-tested, and animals still found to be IFN-γ positive should be removed for pathological and microbiological analysis.

1.2 It would be of value to re-assess cut-offs in respect to different specificity and sensitivity levels by using data from confirmed bovine TB cases in conjunction with the data from negative animals. We, therefore, recommend including data from culture-confirmed animals obtained in the national field trial and 'ad hoc' usage in ROC analysis.

2. Test implementation and possible roll-out:

This test is more complex in its operation than conventional serology assays. To take this into account, we recommend the following:

2.1 Highly descriptive and detailed SOPs need to be drafted and released, with particular emphasis on exclusion criteria for test failures and on how to interpret the test in respect to positive or negative test outcomes (NB. This has now been completed).

2.2 To allow processing of larger sample numbers, a program should be designed as part of the laboratory information management system (LIMS) that will include exclusion and interpretation criteria to flag up automatically potential test failures and also to help test interpretation (NB. This is currently underway).

2.3 Staff undertaking BOVIGAM® testing have to be thoroughly trained before they undertake testing.

2.4 Regular and frequent visits to testing laboratories should be made to ensure strict adherence to SOPs. We further recommend regular re-training sessions.

2.5 Quality control: We recommend that parallel samples are taken regularly and frequently for testing both in the regular testing laboratory and at a 'reference laboratory'.

2.6 To ensure high quality and consistency of training and quality control, we recommend that the activities described in 2.1 to 2.5 to be undertaken by a single 'authority', i.e. a team associated with research activities within the TB Research Group, VLA Weybridge.

2.7 We also recommend that any roll-out of the test into the field should encompass continued monitoring of its performance to allow further assessment and refinement (for example by the definition of 'regionalized cut-off values). This activity should also be undertaken by research scientists within the TB Research Group, most conveniently by the same team responsible for the activities described in 2.1 to 2.5.

3. Recommendations for test application and interpretation

3.1 Our data suggested that the highest test specificity (in excess of 99%) could be achieved by using tuberculin and ESAT-6/CFP-10 in combination, i.e. only double-positive animals are considered positive for bovine TB. Therefore, we recommend using this interpretation of the test when specificity is a premier concern, e.g. when attempting to resolve suspected chronic non-specific skin test reactions (NSR). However, as such an interpretation will also result in decreased sensitivity; we do not recommend its application in situations where maximum sensitivity is required.

3.2 Animals that tested positive with ESAT-6/CFP-10 yet were tuberculin negative: More research is required to determine the status of such animals, in particular to determine

whether such animals/herds occur in geographic clusters, and to attempt to culture mycobacterial species from post-mortemed animals from such herds. However, at the moment such animals should not be classified as TB positive, and results of the comparison between PPD-B and PPD-A should be used to interpret results.

7. References

1. *Ameni, G., H. Miorner, F. Roger, and M. Tibbo. 2000. Comparison between comparative tuberculin and gamma-interferon tests for the diagnosis of bovine tuberculosis in Ethiopia. Trop Anim Health Prod 32:267-76.*

2. *Arend, S. M., P. de Haas, E. Leyten, I. Rosenkrands, L. Rigouts, P. Andersen, W. Mijs, J. T. van Dissel, and D. van Soolingen. 2005. ESAT-6 and CFP-10 in clinical versus environmental isolates of Mycobacterium kansasii. J Infect Dis 191:1301-10.*

3. *Brosch, R., S. V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, L. M. Parsons, A. S. Pym, S. Samper, D. van Soolingen, and S. T. Cole. 2002. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Natl Acad Sci U S A 99:3684-9.*

4. *Buddle, B. M., A. R. McCarthy, T. J. Ryan, J. M. Pollock, H. M. Vordermeier, R. G. Hewinson, P. Andersen, and G. W. de Lisle. 2003. Use of mycobacterial peptides and recombinant proteins for the diagnosis of bovine tuberculosis in skin test-positive cattle. Vet Rec 153:615-20.*

5. *Buonavoglia, D., M. Corrente, R. Moscoa, A. Schiavo, and T. Corsalini. 1995. La diagnosi di tubercolosi bovina: Risultate preliminari sull'impegio del γ-interferon test. Il Progresso Veterinario 23:770 -0772.*

6. *Cagiola, M., F. Feliziani, G. Severi, P. Pasquali, and D. Rutili. 2004. Analysis of possible factors affecting the specificity of the gamma interferon test in tuberculosis-free cattle herds. Clin Diagn Lab Immunol 11:952-6.*

7. *Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, and et al. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393:537-44.*

8. *Collins, J. D. 2002. The control of tuberculosis in cattle: An Irish View. Cattle Practice 10:223 - 229.*

9. *de la Rua-Domenech, R., A. T. Goodchild, H. M. Vordermeier, R. G. Hewinson, K. H. Christiansen, and R. S. Clifton-Hadley. 2006. Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. Res Vet Sci.*

10. *Doherty, M. L., M. L. Monaghan, H. F. Bassett, and P. J. Quinn. 1995. Effect of a recent injection of purified protein derivative on diagnostic tests for tuberculosis in cattle infected with Mycobacterium bovis. Res Vet Sci 58:217-21.*

11. *Domingo, M., E. Liebana, M. Vilafranca, A. Aranaz, D. Vidal, N. Prats, A. Mateos, J. Casal, and L. Dominguez. 1995. A field evaluation of the interferon-gamma assay and the intradermal tuberuclin test in diary cattle in Spain, p. 3040-306. In F. Griffin and G. de Lisle (ed.), Tuberculosis in Wildlife and Domestic Animals. Universtity of Otago Press, Dunedin*

12. *Garnier, T., K. Eiglmeier, J. C. Camus, N. Medina, H. Mansoor, M. Pryor, S. Duthoy, S. Grondin, C. Lacroix, C. Monsempe, S. Simon, B. Harris, R. Atkin, J. Doggett, R. Mayes, L. Keating, P. R. Wheeler, J. Parkhill, B. G. Barrell, S. T. Cole, S. V. Gordon, and R. G. Hewinson. 2003. The complete genome sequence of Mycobacterium bovis. Proc Natl Acad Sci U S A 100:7877-82.*

13. *Gonzalez Llamazares, O. R., C. B. Gutierrez Martin, D. Alvarez Nistal, V. A. de la Puente Redondo, L. Dominguez Rodriguez, and E. F. Rodriguez Ferri. 1999. Field evaluation of the single intradermal cervical tuberculin test and the interferon-gamma assay for detection and eradication of bovine tuberculosis in Spain. Vet Microbiol 70:55-66.*

14. *Gormley, E., M. B. Doyle, K. McGill, E. Costello, M. Good, and J. D. Collins. 2004. The effect of the tuberculin test and the consequences of a delay in blood culture on the sensitivity of a gamma-interferon assay for the detection of Mycobacterium bovis infection in cattle. Vet Immunol Immunopathol 102:413-20.*

15. *Lauzi, S., D. Pasotto, M. Amadori, I. L. Archetti, G. Poli, and L. Bonizzi. 2000. Evaluation of the specificity of the gamma-interferon test in Italian bovine tuberculosis-free herds. Vet J 160:17-24.*

16. *Lilenbaum, W., J. C. Schettini, G. N. Souza, E. R. Ribeiro, E. C. Moreira, and L. S. Fonseca. 1999. Comparison between a gamma-IFN assay and intradermal tuberculin test for the diagnosis of bovine tuberculosis in field trials in Brazil. Zentralbl Veterinarmed B 46:353-8.*

17. *McCorry, T., A. O. Whelan, M. D. Welsh, J. McNair, E. Walton, D. G. Bryson, R. G. Hewinson, H. M. Vordermeier, and J. M. Pollock. 2004. Shedding of Mycobacterium bovis in the nasal mucus of cattle experimentally infected with tuberculosis by the intranasal and intratracheal routes. Vet. Rec. in press.*

18. *Monaghan, M. L., P. J. Quinn, A. P. Kelly, K. McGill, C. McMurray, K. O'Crowley, H. F. Bassett, E. Costello, F. Quigley, J. S. Rother, P. R. Wood, and D. M. Collins. 1997. A pilot trial to evaluate the γ-interferon assay for the detection of Mycobacterium bovis infected cattle under Irish conditions. Irish Vet. J. 50:229 - 232.*

19. *Neill, S. D., D. G. Bryson, and J. M. Pollock. 2001. Pathogenesis of tuberculosis in cattle. Tuberculosis (Edinb) 81:79-86.*

20. *Neill, S. D., J. Cassidy, J. Hanna, D. P. Mackie, J. M. Pollock, A. Clements, E. Walton, and D. G. Bryson. 1994. Detection of Mycobacterium bovis infection in skin test-negative cattle with an assay for bovine interferon-gamma. Vet. Rec. 135:134-5.*

21. *Neill, S. D., J. Hanna, D. P. Mackie, and T. G. Bryson. 1992. Isolation of Mycobacterium bovis from the respiratory tracts of skin test-negative cattle. Vet. Rec. 131:45-7.*

22. *North, R. J., and Y. J. Jung. 2004. Immunity to tuberculosis. Annu Rev Immunol 22:599-623.*

23. *Palmer, M. V., W. R. Waters, T. C. Thacker, R. Greenwald, J. Esfandiari, and K. P. Lyashchenko. 2006. Effects of different tuberculin skin-testing regimens on gamma interferon and antibody responses in cattle experimentally infected with Mycobacterium bovis. Clin. Vaccine Immunol. 13:387 - 394.*

24. *Pollock, J. M., R. M. Girvin, K. A. Lightbody, R. A. Clements, S. D. Neill, B. M. Buddle, and P. Andersen. 2000. Assessment of defined antigens for the diagnosis of bovine tuberculosis in skin test-reactor cattle. Vet Rec 146:659-65.*

25. *Pollock, J. M., J. McNair, M. D. Welsh, R. M. Girvin, H. E. Kennedy, D. P. Mackie, and S. D. Neill. 2001. Immune responses in bovine tuberculosis. Tuberculosis (Edinb) 81:103-7.*

26. *Ritacco, V., B. Lopez, I. N. De Kantor, L. Barrera, F. Errico, and A. Nader. 1991. Reciprocal cellular and humoral immune responses in bovine tuberculosis. Res. Vet. Sci. 50:365-7.*

27. *Rothel, J. S., S. L. Jones, L. A. Corner, J. C. Cox, and P. R. Wood. 1992. The gamma-interferon assay for diagnosis of bovine tuberculosis in cattle: conditions affecting the production of gamma-interferon in whole blood culture. Aust. Vet.J. 69:1-4.*

28. *Ryan, T. J., B. M. Buddle, and G. W. De Lisle. 2000. An evaluation of the gamma interferon test for detecting bovine tuberculosis in cattle 8 to 28 days after tuberculin skin testing. Res Vet Sci 69:57-61.*

29. *Vordermeier, H. M. 2002. Final report, project SE3005. CSG16.*

30. *Vordermeier, H. M., A. Whelan, P. J. Cockle, L. Farrant, N. Palmer, and R. G. Hewinson. 2001. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin Diagn Lab Immunol 8:571-8.*

31. *Whelan, A. O., M. Coad, Z. A. Peck, D. Clifford, R. G. Hewinson, and H. M. Vordermeier. 2004. Influence of skin testing and overnight sample storage on blood-based diagnosis of bovine tuberculosis. Vet Rec 155:204-6.*

32. *Whipple, D. L., C. A. Bolin, A. J. Davis, J. L. Jarnagin, D. C. Johnson, R. S. Nabors, J. B. Payeur, D. A. Saari, A. J. Wilson, and M. M. Wolf. 1995. Comparison of the sensitivity of the caudal fold skin test and a commercial gamma-interferon assay for diagnosis of bovine tuberculosis. Am. J. Vet. Res. 56:415-419.*

33. *Whipple, D. L., M. V. Palmer, R. E. Slaughter, and S. L. Jones. 2001. Comparison of purified protein derivatives and effect of skin testing on results of a commercial gamma interferon assay for diagnosis of tuberculosis in cattle. J Vet Diagn Invest 13:117-22.*

34. *Wood, P. R., L. A. Corner, J. S. Rothel, C. Baldock, S. L. Jones, D. B. Cousins, B. S. McCormick, B. R. Francis, J. Creeper, and N. E. Tweddle. 1991. Field comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. Aust. Vet. J. 68:286-290.*

35. *Wood, P. R., L. A. Corner, J. S. Rothel, J. L. Ripper, T. Fifis, B. S. McCormick, B. Francis, L. Melville, K. Small, K. de Witte, and et al. 1992. A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. Vet. Microbiol. 31:71-79.*

36. *Wood, P. R., and S. L. Jones. 2001. BOVIGAM®: an in vitro cellular diagnostic test for bovine tuberculosis. Tuberculosis (Edinb) 81:147-55.*

8. Appendices

Appendix 1: Tables and Figures

Table A1: Examples of published studies assessing the sensitivity and specificity of the BOVIGAM® assay using bovine and avian PPD.

Country	IFN-γ Sensitivity (%)	Specificity (%)	Skin test Sensitivity (%)	Specificity (%)	Reference
Australia	84.4	98	65.6[a]	ND	(34)
Australia	81.8	99.1	68.1[a]	96.7 [a]	(35)
USA	80.9	ND	80.4[a]	ND	(32)
Brazil	100	ND	88.3[a]	ND	(16)
Spain	84.9	ND	80.2[a]	ND	(13)
Spain	87.6	ND	75.3[a]	ND	(11)
Northern Ireland*	89.3	99.2	ND	ND	(24)
New Zealand*	85	93	ND	ND	(28)
New Zealand*	94.6	(73.6[d])	ND	(0[ac])	(4)
Italy	NT	88.4	ND	ND	(15)
Italy*	96.6	98	ND	ND	(5)
Italy	NT	97.3	ND	96.8[b]	(6)
Ethiopia	95.5	87.7	90.9[b]	100[b]	(1)
Romania	92.5	ND	81.8	ND	(36)
Eire*	84.2	96.2	ND	ND	(18)
Eire*	88.5	NT	59.9/74.3[b]**	ND	(8)
GB*	88.2	92	ND	ND	(30)
GB*	89.7	96.6	ND	ND	(29)
Median	**88.3**	**96.6**	**77.7[a]**	**ND**	
(range)	**(80.9-100)**	**(87.7-99.2)**	**(65.6-88.3)[a]**		

A number of different cut-off values and interpretation protocols were applied in the different trials. However, results in all studies are based on the comparison of responses to avian and bovine PPD.

*Post-skin test assays. **Standard/severe interpretation of the skin test.

[a]Single intradermal test (single cervical intradermal tuberculin test or caudal fold test).

[b]Comparative cervical tuberculin test.

[c]Study concentrated on false-positive skin test positive animals, i.e. specificity of skin test is 0 % in this cohort, data not used to determine median specificity.

[d]Not included in median determinations. ND, not determined.

Table A2. Increase in overall sensitivity by using SICCT and BOVIGAM® test in parallel.

Study	*IFN-γ (%)*	*ST (%)*	*ST + IFN-γ (%)*	*Reference*
Wood, 1991	93.6	65.6	95.2	(34)
Whipple, 1995	73	80.4	90.7	(32)
Gonzalez, 1999	84.9	80.2	92.9	(13)
Collins, 2002	88.5	74.3*	97.0*	(8)
Goodchild, 2004*	70.0	65.0	88.0	Unpublished data

*Severe interpretation of skin test. **Data from herd that was de-populated.

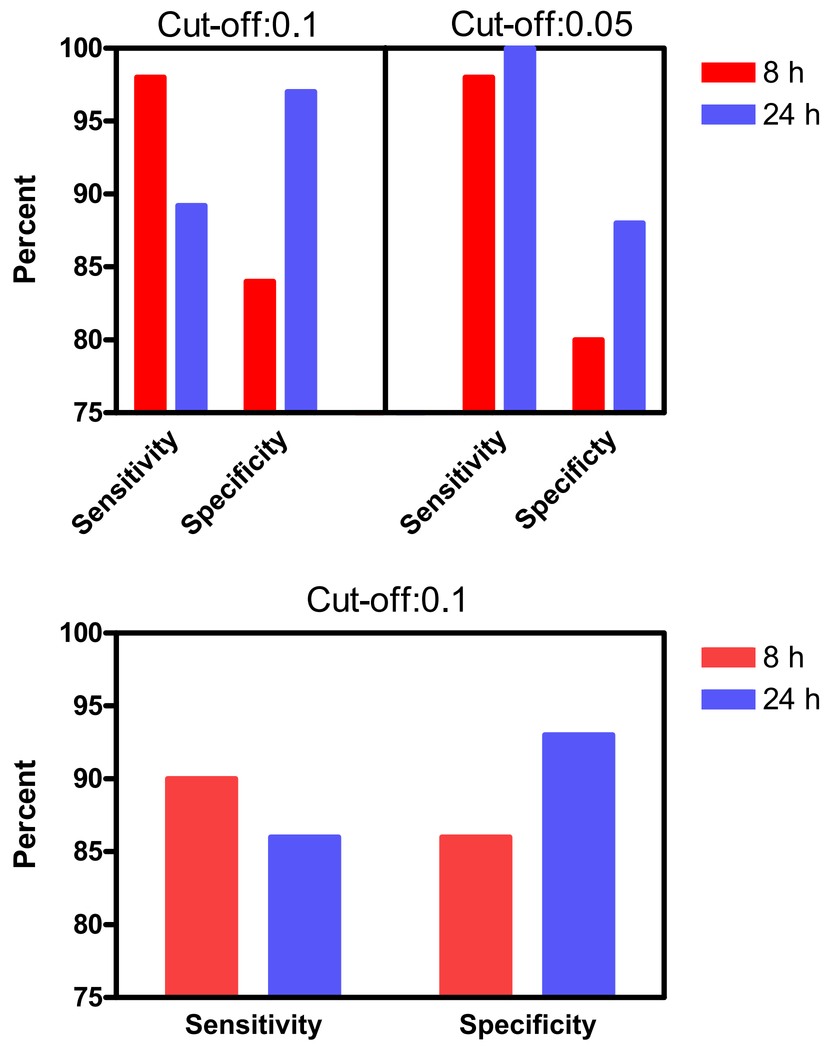

Figure A1. **Comparison of specificity and sensitivity in relation to blood storage time.**
Upper panel: Results obtained in GB (Vordermeier et al., final report SE2005)
Lower panel: Results reported by Ryan et al. (28).

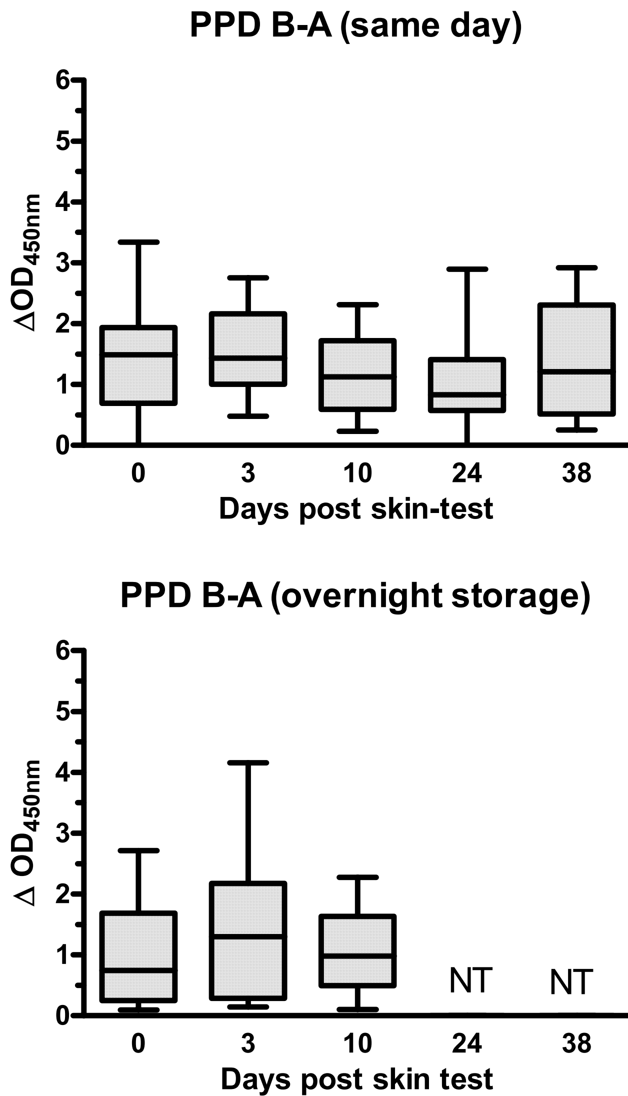

Figure A2. **Interferon-gamma responses in relation to sampling post-skin test (SICCT).**
Upper panel. Samples (n=18) were taken at indicated intervals post-SICCT application and tested on the day of sampling.
Lower panel: Same samples as above were left overnight before testing in the BOVIGAM® assay.
Responses in both panels are expressed as box-whiskers plots of OD450 with PPD-B minus OD450 with PPD-A (PPD B-A).

Appendix 2.

Infection biology of bovine tuberculosis in cattle in relation to diagnostic tests

It is generally accepted that cell-mediated immune (CMI) responses are the principal and earliest immune responses to develop after infection with *M. bovis*, the causative agent of bovine tuberculosis (Figure A3, (19, 25, 26)). Antibody responses develop considerably later than CMI responses (Fig. A3) and it is therefore not surprising that assays based on measuring CMI, like the tuberculin skin test and the BOVIGAM® IFN-γ assay have been used extensively for the diagnosis of bovine tuberculosis (BTB) in cattle.

To better understand the principles of the tuberculin skin and the BOVIGAM® IFN-γ tests, it is useful to discuss these assays in the context of the pathogenesis of BTB. Bovine tuberculosis in cattle primarily is presented as visible lesions in the lungs and associated lymph nodes and/or in the lymph nodes of the head. *M. bovis* can also be cultured from tissues with or without visible lesions. As *M. bovis*, is closely related to the pathogen causing human tuberculosis, *M. tuberculosis* (3, 7, 12) it has been proposed that the principles of *M. bovis* pathogenesis in cattle follows closely that of *M. tuberculosis* in humans (22).

Humans in contact with TB patients will be ***exposed*** *to M. tuberculosis* as will be cattle in a herd with confirmed bovine TB. A certain proportion (10 - 30 % in the case of humans) of exposed individuals will actually become **infected** (as defined by acquired delayed type hypersensitivity and/or IFN-γ responses). About 5 % of infected humans develop **disease** within 1 year of infection (*primary tuberculosis*), whilst 95 % of infected individuals do not. Such individuals – i.e. being tuberculin skin test positive and showing IFN-γ responses *in vitro* to antigens such as ESAT-6 but not presenting with clinical or radiological signs of disease -

are classified as **latently** infected. However, 5-10% of latently infected humans develop clinical tuberculosis during their lifetime through re-activation (*re-activation tuberculosis*). Thus, the population of latently infected humans presents a huge reservoir of *M. tuberculosis* infection. **Although exposed, latent and diseased states are likely to be equally applicable to bovine tuberculosis in cattle, the proportions of these disease states occurring in cattle are not known to date even if it is likely that the proportion of latent infections will be considerably lower in bovine tuberculosis than in human tuberculosis. However, the argument that latently infected individuals (culture-negative NVL, skin test reactors for example) constitute a continuous and unpredictable source of re-infection, is equally valid for cattle as it is for human TB**.

This concept of infection without signs of disease (latency), as defined in the previous paragraph, versus detectable disease has important implications for the interpretation of tests like the IFN-γ test or the skin test (see also figure A4): In early stages post-infection, or in latently infected cattle, a period will occur when *M. bovis* appears to be absent, because the bacillary load is not large enough to be cultured. In addition, the pathological changes caused by the bacilli are not yet profound enough to be detectable during routine abattoir inspection. Due to this latency period between infection and the development of detectable signs of bovine tuberculosis, cellular immune responses will be detectable earlier than the pathological changes caused by the disease (e.g. visible lesions), and before the bacterial loads exceed the numbers necessary for the detection of *M. bovis* from tissue samples by culture (Fig. A3). This has two consequences: firstly, immuno-diagnostic assays of cellular immunity (which includes the IFN-γ test as well as the tuberculin skin test), by detecting infection rather than disease, can be very sensitive in identifying *M. bovis* infected cattle. Secondly, at abattoir inspections, a proportion of these identified animals will not have visible lesions, nor can *M. bovis* be subsequently cultured from tissue samples. To designate these animals as 'false-

positive' is highly inappropriate as it is also obvious that such animals can harbour the bacilli, and when the disease has progressed further, may become infectious to other cattle.

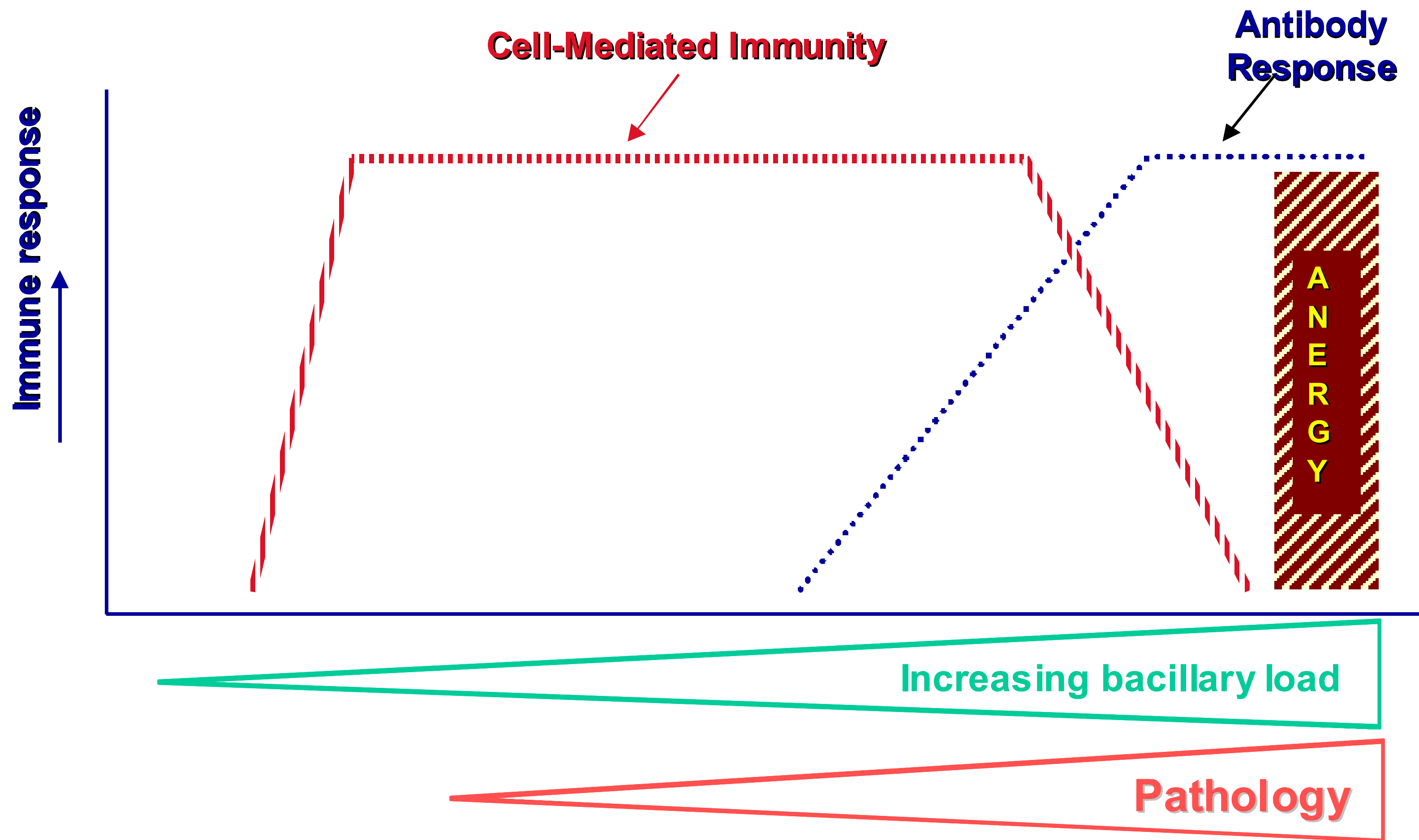

Figure A3. **Development of immune responses in cattle following *M. bovis* infection: The spectrum of disease**

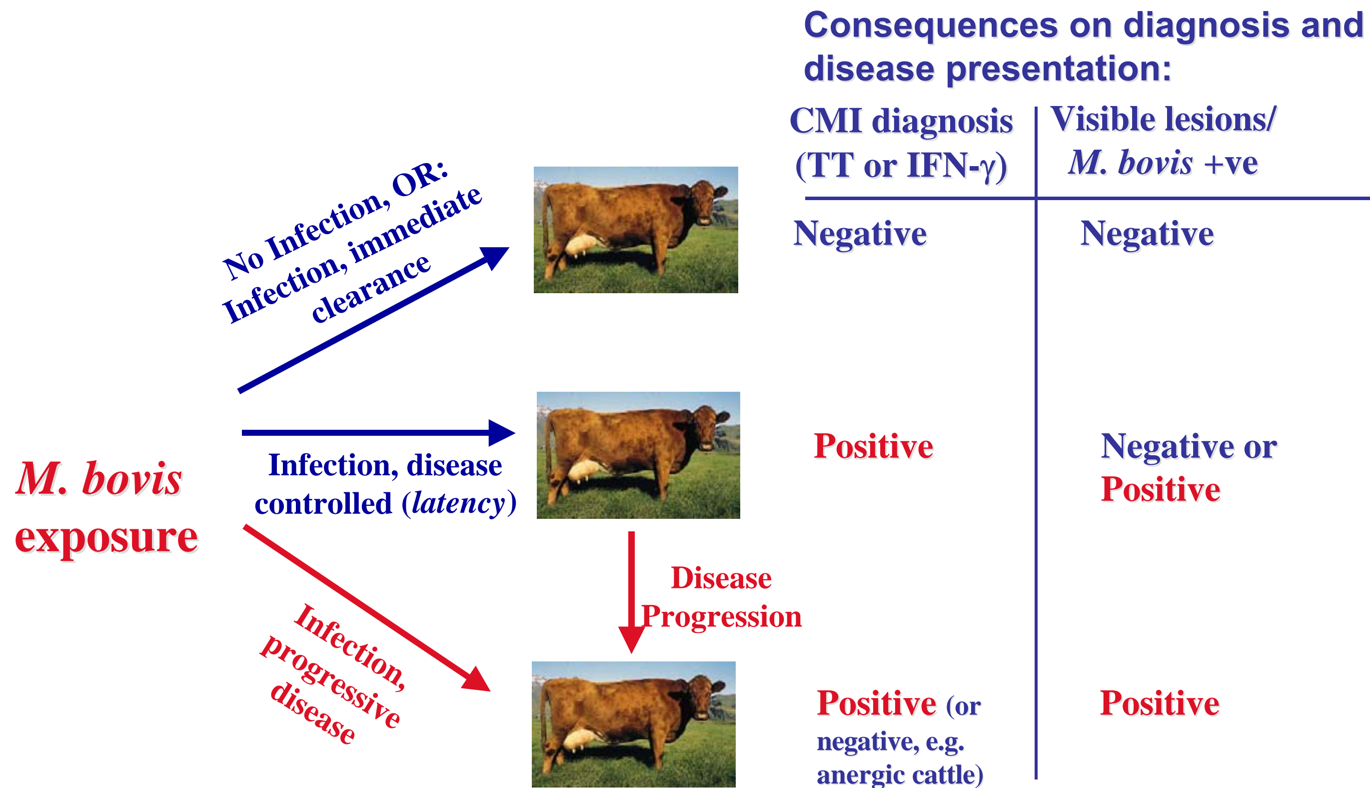

Figure A4. **Biology of bovine tuberculosis and the consequences on the outcome of diagnostic tests.** Inspired by Dannenberg, AM. Pathogenesis of pulmonary Mycobacterium bovis infection: basic principles established by the rabbit model. 2001. Tuberculosis 81; 87-96. **Please note that the degree of latency in cattle is unknown.**