OIE Procedure for Registration of Diagnostic Kits
Validation Studies Abstract

Name of the diagnostic kit: Enferplex Bovine TB antibody test
Manufacturer: Enfer Scientific ULC
OIE Approval number: 20190113
Date of Registration: May 2019
Disease: Bovine tuberculosis
Pathogen Agent: *Mycobacterium bovis*
Type of Assay: Indirect chemiluminescent multiplex ELISA

Purpose of Assay:
Certified by the OIE as fit for the detection of antibody to *Mycobacterium bovis* in cattle serum samples, to be used as an ancillary test in conjunction with other methods for serological prevalence surveys, or diagnosis and management of *M. bovis* infection within herds, for the following purposes:

1. To confirm, but not negate, diagnosis of suspect or clinical cases, including confirmation of positive screening tests in individual animals and in herds with infection prevalence ranging from very low to high, based on detection of antibodies in bovine serum.
2. To detect *Mycobacterium bovis* infected animals not positive by single intradermal comparative cervical tuberculin (SICCT) or interferon gamma release assay (IFNɣ) tests, based on detection of antibodies in bovine serum.
3. To confirm, but not negate, infection in animals giving inconclusive reactions in the SICCT, based on detection of antibodies in bovine serum.
4. As a screening test, to identify animals most likely to have visible lesions (VL) by scoring the number of *M. bovis* antigens recognised by seropositive animals with bovine tuberculosis.

Species and Specimens:
This test has been validated and approved for testing serum samples from cattle, as noted above.
Regarding intended use 4 above, during the first 5 years of registration, additional data will be required to better qualify and categorise the relationship between the number of *M. bovis* antigens and the likelihood of visible lesions.
This test is also provisionally approved for testing milk samples from cattle as a herd screening test or as a supplemental confirmatory test for use in individual animals, when used in conjunction with other methods for diagnosing and managing *M. bovis* infection.

1. Information on the kit

Please refer to the kit insert available on the OIE Registry web page or contact manufacturer at:

Enfer Scientific ULC
Unit T, M7 Business Park, Newhall, Naas, Kildare, Ireland.
Web: https://www.enfergroup.com/
Email: info@enfergroup.com
Tel: 00353 45 983800
Fax: 00353 45 983801

2. Summary of validation studies

Analytical characteristics

Repeatability using serum

To determine the within-run, between-run, and between-batch variation, three categories of sera were used to: one serum sample negative against all 11 antigens; one serum dilution for each antigen giving strong positivity; one serum dilution for each antigen giving weak positivity. The samples were run in quadruplicate over 20 runs, split between 2 days and 2 operators. The mean, standard deviation (SD) and coefficient (CV) were calculated. The variation in signal-to-cutoff (S/CO) ratios within-run, between run, and between batches is shown in Table 1. The CVs ranged between 1.3 and 6.5%, and mean values did not exceed 2 SDs over 20 runs of the test.

Table 1. Variation in S/CO ratios within-run, between run, and between batches using serum samples

Test	Sample	%CV
Within run	**Strong positive**	**1.3 – 3.3**
	Weak positive	**4.0 – 6.2**
	Negative	**Undetermined due to S/CO ratios being around or below zero**
Between run	**Strong positive**	**1.3 – 3.8**
	Weak positive	**4.0 – 5.9**
	Negative	**Undetermined due to S/CO ratios being around or below zero**
Between batch	**Strong positive**	**1.3 – 3.1**
	Weak positive	**3.6 – 6.5**
	Negative	**Undetermined due to S/CO ratios being around or below zero**

Repeatability (Milk):

To determine the within-run and between-run repeatability, three categories of milk sample were used: one serum sample negative against all 11 antigens; one serum dilution for each antigen giving strong positivity; one serum dilution for each antigen giving weak positivity. The samples were run in quadruplicate over 20 runs, split between 2 days and 2 operators. The mean, standard deviation (SD) and coefficient (CV) were calculated.

The variation in S/CO ratios within-run and between run is shown in Table 2. The CVs ranged between 1.4 and 11.1%, and mean values did not exceed 2 SDs over 20 runs of the test.

Table 2. Variation in S/CO ratios within-run and between run

Test	Sample	%CV
Within run	**Strong positive**	**1.4 – 3.4**
	Weak positive	**3.8 – 9.8**
	Negative	**Undetermined due to S/CO ratios being around or below zero**
Between run	**Strong positive**	**1.4 – 4.1**
	Weak positive	**4.3 – 11.1**
	Negative	**Undetermined due to S/CO ratios being around or below zero**

The Enferplex Bovine TB antibody test thus shows acceptable within-run, between run, and between batch repeatability for the detection of anti-*M. bovis* antibodies in serum and milk across the range of the assay.

Analytical specificity

Blood samples were obtained from 8 animals experimentally infected with *M. kansasii* pre-infection and at 18 weeks post-infection. All animals were negative against all antigens except for one animal that gave a positive result against one antigen.

Blood samples were also obtained from naturally infected animals that were either positive for *M*. *avium subsp. paratuberculosis (*MAP) culture (n = 258), MAP antibody positive determined by a commercial ELISA (251), or both (n = 329). The results of testing these sera in the Enferplex Bovine TB antibody test gave relative specificity estimates of 98.1% and 99.2% (MAP culture positive) and 98.0% and 100% (MAP antibody positive) using the high sensitivity and high specificity settings of the test respectively. These relative specificity estimates close to the overall specificity of the Enferplex Bovine TB antibody test in Bovine TB free animals (98.4%) using the high sensitivity setting of the test, and indicate a lack of cross-reactivity between MAP antibodies and the *M. bovis* antigens used in the Enferplex Bovine TB antibody test. The data indicate that the kit is very specific with respect to cross reactions with non-tuberculosis environmental organisms such as *M. kansasii* and MAP.

Analytical sensitivity using serum

Six animals were infected experimentally with *M. bovis* and blood samples obtained pre-infection and post infection at 3, 7, 11, and 12 weeks. The results show that the kit detects anti-*M. bovis* antibody in 5/6 animals within 7 weeks of experimental infection with *M. bovis*. In 10 animals infected experimentally with *M. tuberculosis*, blood samples were obtained pre-infection and at 4, 6, 7, 8, and 9 weeks post-infection. Antibodies were detected within 4 weeks of infection in 2/10 animals. The kit, thus, detects infection early in the infection process. Lower levels of antibody were observed after *M. tuberculosis* compared to *M. bovis* experimental infection. This may reflect the fact that *M. tuberculosis* is less virulent in cattle compared to *M. bovis.*

Analysis of analytical sensitivity of the Enferplex Bovine TB antibody test was also determined using endpoint titration of a strong positive serum sample. The results show that endpoint titres for individual antigens ranged from 1:3200 – 1:512000 across the 11 antigens in the test.

Analytical sensitivity using milk

Analysis of analytical sensitivity of the Enferplex Bovine TB antibody test was also determined using endpoint titration of a strong positive milk sample. The results show that endpoint titres for individual antigens ranged from 1:160 – 1:2560 across the 11 antigens in the test.

Diagnostic Characteristics

Threshold determination

The thresholds for individual antigens were determined empirically, targeting overall specificity at 98% using the high sensitivity setting of the test. The threshold for overall assay positivity was set based on a 2-antigen rule, whereby the Relative Light Units signals from two or more antigens need to be above their individual antigen thresholds for the sample to be registered as “positive” (Whelan et al, 2008). Thresholds for individual antigens were set between 95.1 – 99.8% specificity based on true negative reference sera to give a “High Sensitivity” setting using the 2-antigen rule. Thresholds for individual antigens were set between 97.3 – 99.9% specificity based on true negative reference sera to give a “High Specificity” setting for the test using the two-antigen rule. The ‘Enferplex Bovine TB antibody test Macro’ provided, automatically calculates the results for high specificity and high sensitivity.

Diagnostic sensitivity (DSn) and specificity (DSp) estimates with 95% confidence limits (CI)

The performance levels indicated below were based on multiple batches of the Enferplex Bovine TB antibody test and reflect the biological diversity with respect to kit components (recombinant antigens, buffers, and conjugates, positive and negative controls.

Diagnostic specificity **(DSp) - serum**

Relative diagnostic specificity was estimated using animals from the United Kingdom (UK), Ireland (IE), Norway (NO), Switzerland (CH), Liechtenstein (LI), France (FR) and United States of America (USA) that were deemed to be free of Bovine TB (Table 3).

Table 3. Diagnostic specificity of Enferplex Bovine TB antibody test at the high sensitivity and high specificity settings in samples from Bovine TB free animals from the EU and USA.

Animal category under evaluation	Statistical variable	Target Species – cattle High Sensitivity	Target Species – cattle High Specificity
Bovine TB free animals UK, IE, CH/LI, NL, ES, NO and USA Non-boosted	N DSp CI	**4258** **98.4%** **98.0-97.8**	**4258** **99.7%** **99.5-99.8**
Bovine TB free animals FR Boosted	N DSp CI	**161** **100%** **-**	**161** **100%** **-**

The results show that the Enferplex Bovine TB antibody test had a DSp of 98.4% and 99.7% at the high sensitivity and high specificity settings respectively in Bovine TB free animals not boosted through prior PPD injection ('non boosted'). In Bovine TB free animals boosted through prior PPD injection ('Boosted'), the DSp was 100% at both sensitivity settings.

Diagnostic sensitivity **(DSn)**

Serum samples

Diagnostic sensitivity was estimated using serum samples from *M. bovis* culture positive animals, SICCT or CFT positive animals, and IFNɣ test positive animals from the UK, IE, Italy (IT) and USA.

Table 4. Diagnostic sensitivity of Enferplex Bovine TB antibody test at the high sensitivity and high specificity settings in 478 samples from *M. bovis* culture positive animals

Animal category under evaluation	Statistical variable	Target Species – cattle High Sensitivity	Target Species – cattle High Specificity
Positive by *M. bovis* culture Boosted samples UK, IE	N DSn CI	**208** **94.2%** **91.1-97.4**	**208** **94.2%** **91.1-97.4**
Positive by *M. bovis* culture Non-boosted samples UK, USA	N DSn CI	**182** **75.8%** **69.6-82.0**	**182** **71.4%** **64.9-78.0**
Positive by *M. bovis* culture Unknown boost status IT	N DSn CI	**88** **80.7%** **72.4-89.0**	**88** **75.0%** **66.0-84.1**
Positive by *M. bovis* culture All samples UK, IE, USA, IT	N DSn CI	**478** **84.7%** **81.2-87.7**	**478** **82.0%** **78.3-85.0**

The results show that the Enferplex Bovine TB antibody test at both the high sensitivity and high specificity settings has a high sensitivity in *M. bovis* culture positive animals. The overall sensitivity of the Enferplex Bovine TB antibody test in *M. bovis* positive animals was 84.7% using the high sensitivity setting. Using the high specificity setting of the Enferplex Bovine TB antibody test, diagnostic sensitivity was 82.0%. Samples pre-boosted with PPD had a higher sensitivity (94.2%) at both the high sensitivity and high specificity settings. In 'non-boosted' samples, the figures were significantly lower at 75.8% and 71.4% respectively.
The Enferplex Bovine TB antibody test was assessed using the SICCT or CFT as the reference standards and the results are shown in Table 5.

Table 5. Relative diagnostic sensitivity of the Enferplex Bovine TB antibody test at the high sensitivity and high specificity settings in SICCT or CFT positive animals.

Animal category under evaluation	Statistical variable	Target Species – cattle High Sensitivity	Target Species – cattle High Specificity
Positive by SICCT Boosted UK, IE	N RSn CI	**2076** **92.6%** **91.5-93.8**	**2076** **89.6%** **88.2-90.9**
Positive by CFT Non-boosted USA	N RSn CI	**90** **82.2%** **74.3-90.1**	**90** **75.6%** **66.7-84.4**
Positive SICCT and CFT All samples UK, IE, USA	N RSn CI	**2166** **92.3%** **91.2-93.4**	**2166** **89.0%** **87.6-90.3**

The overall relative sensitivity of the Enferplex Bovine TB antibody test against the SICCT or CFT was 92.3% using the high sensitivity setting. Using the high specificity setting of the Enferplex Bovine TB antibody test, relative sensitivity was 89.0%. Samples pre-boosted with PPDb had a higher relative sensitivity of 92.6% and 89.6% at the high sensitivity and high specificity settings respectively. In 'non-boosted' samples the figures were significantly lower at 82.2% and 75.6% respectively.

The Enferplex Bovine TB antibody test was assessed for its ability to detect animals that have given two consecutive inconclusive reactor (2 x IR) results in Short Interval Testing in the UK (deemed to be 'reactors' in the UK). The results of analysing the relationship between 2 x IR SICCT reactors and antibody responses are shown in Table 6.

Table 6. Relative diagnostic sensitivity of Enferplex Bovine TB antibody test using samples from UK animals with 2 x IR status as reference standards

Animal category under evaluation	Statistical variable	Target Species – cattle High Sensitivity	Target Species – cattle High Specificity
2 x IR Boosted UK	N RSn CI VL or *M. bovis* positive (%)	**187** **87.7%** **83.0-92.4** **13 (7.9%)**	**187** **80.2%** **74.5-85.9** **13 (8.7%)**
1 x IR Boosted UK	N RSn CI VL or *M. bovis* positive	**111** **73.9%** **65.7-82.0** **10 (12.2%)**	**111** **71.2%** **62.7-79.6** **10 (12.7%)**

The relative sensitivity was 87.7% and 80.2% using the high sensitivity and high specificity cut-offs respectively. Using the presence of VL or culture of *M. bovis* as positive reference standards showed that 7.9% and 8.7% of these Enferplex Bovine TB Antibody test positive animals were positive at the high sensitivity and high specificity settings respectively.
For animals with 1 x IR status (not deemed to be 'reactors' in the UK) the relative sensitivity was 73.9% and 71.2% at the high sensitivity and high specificity settings respectively. The results showed that 12.2% and 12.7% of Enferplex Bovine TB Antibody test positive animals had VL or were *M. bovis* culture positive using the high sensitivity and high specificity settings respectively. The Enferplex Bovine TB Antibody test thus detects infected animals that register as inconclusive in the SICCT.

The Enferplex Bovine TB antibody test was assessed using the IFN□ test as the reference standard and the results are shown in Table 7.

Table 7. Relative diagnostic sensitivity of Enferplex Bovine TB antibody test at the high sensitivity and high specificity settings using in IFNγ test positive animals from UK, IE, IT and USA.

Animal category under evaluation	Statistical variable	Target Species – cattle High Sensitivity	Target Species – cattle High Specificity
Positive by IFNγ Boosted UK, IE	N RSn CI	**1220** **86.7%** **84.8-88.6**	**1220** **82.7%** **80.6-84.8**
Positive by IFNγ Non-boosted UK, USA	N RSn CI	**1317** **45.7%** **43.0-48.4**	**1317** **39.6%** **39.6-42.2**
Positive by IFNγ Boost status unknown IT	N RSn CI	**110** **69.1%** **60.4-77.3**	**110** **66.4%** **57.5-75.2**
Positive by IFNγ All samples UK, IE, IT, USA	N RSn CI	**2537** **65.4%** **63.6-67.3**	**2537** **60.3%** **58.4-62.2**
Negative by IFNγ	N RSp CI	**No direct comparison performed on IFNγ negatives from negative herds**	

The results show that the overall relative sensitivity of the Enferplex Bovine TB antibody test using samples from IFNɣ positive animals was 65.4% at the high sensitivity setting and 60.3% at the high specificity setting. Samples pre-boosted with PPDb had a significantly higher relative sensitivity, 86.7% and 82.7% at the high sensitivity and high specificity settings respectively. In 'non-boosted' samples the figures were significantly lower at 45.7% and 39.6% respectively.

SICCT negative or IFNɣ negative animals with lesions typical of Bovine TB
The results of testing samples from SICCT negative, Bovine TB lesion positive animals in the Enferplex Bovine TB antibody test are shown in Table 8.

Table 8. Relative sensitivity of Enferplex Bovine TB antibody test in bTB lesion positive animals that were negative by SICCT of IFNɣ test.

Animal category under evaluation	Statistical variable	Target Species – cattle High Sensitivity	Target Species – cattle High Specificity
SICCT negative, bTB lesion positive Boosted UK, IE	N RSn CI	**137** **88.3%** **82.9-93.7**	**137** **82.5%** **76.1-88.8**
IFNɣ negative, Bovine TB lesion positive Boosted IE	N RSn CI	**57** **82.5%** **72.6-92.3**	**57** **75.4%** **64.3-86.6**

The results show that the relative sensitivity of the Enferplex Bovine TB antibody test in SICCT negative animals with lesions was 88.3% at the high sensitivity setting and 82.5% at the high specificity setting. Similarly, the relative sensitivity of the Enferplex Bovine TB antibody test in IFNɣ test negative animals with lesions was 82.5% at the high sensitivity setting and 75.4% at the high specificity setting. The results thus show that the Enferplex Bovine TB Antibody test detects infected animals that register negative in the SICCT.

Relationship between the number of antigens recognised by antibody and the likelihood of lesions

To investigate the relationship between the numbers of antigens recognised as positive in the Enferplex Bovine TB antibody test, serum samples from 2056 SICCT positive animals were tested and the results correlated with the Bovine TB lesion status at post-mortem.
If the populations of animals are aggregated by the number of antigens that are recognized it can be seen in Table 9 that just under 90% of animals that had 5 or more antigens recognized by the Enferplex Bovine antibody test had visible lesions.

Table 9. Relationship between the number of antigens recognized and visible lesions in 2056 SICCT-positive cattle from the UK and IE

Number of Antigens Recognised	Total number (n = 2056)	Percentage with VL	Percentage of total VL
0 or 1	150	23.3	3.3
2 or more	1906	54.4	96.7
3 or more	1823	55.8	94.9
4 or more	1759	56.6	92.9
5 or more	1670	57.2	89.1
6 or more	1436	59.0	79.1
7 or more	1071	62.7	62.7
8 or more	804	65.5	49.2
9 or more	519	69.5	33.7
10 or more	364	74.2	25.2
11	121	78.5	8.9

Milk samples

The relative diagnostic sensitivity and specificity of the Enferplex Bovine TB antibody test was estimated using milk samples. Paired serum and milk samples from 107 SICCT positive animals and from 1149 true negative reference animals in the UK were tested in the Enferplex Bovine TB antibody test. The results are shown in Table 10.

Table 10. Relative sensitivity of the Enferplex Bovine TB antibody test in milk samples.

Animal category under evaluation	Statistical variable	Target Species – cattle High Sensitivity	Target Species – cattle High Specificity
Positive by SICCT	N RSn CI	**107** **91.6%** **84.8-95.5**	**107** **82.2%** **73.9-88.3**
Negative by SICCT, and/or OTF status and Bovine TB history	N DSp CI	**1149** **99.8%** **99.5-100.0**	**1149** **99.8%** **99.5-100.0**

The results show that the relative sensitivity was 91.6% using the high sensitivity, and 82.2% using the high specificity setting of the test in milk. The specificity was 99.8% using the high sensitivity setting and 99.8% using the high specificity setting of the test.
The results for paired serum and milk were analysed using Spearman's Rank Correlation test. Correlation coefficients obtained for each antigen ranged between 0.8 – 0.95 across all 11 antigens.

Reproducibility

Analytical reproducibility

An evaluation panel of serum samples comprising negative, weak positive and strong positive serum samples were blinded and sent to the 3 laboratories for analytical reproducibility testing. Seven negative samples, 7 weak positive samples, and 7 strong positive samples were tested using two plates from two different kit batches and 1 technician in each laboratory. The results were sent to Enfer Scientific for un-blinding and analysis.
For the analyses, a series of linear mixed effect models were run with kit batch, laboratory, plate and sample taken into account. The results included the overall means, SD, CV, LCL, UCL, and 95%CI, and an estimate of how much variation was due to these variables, and statistical assessment of the differences observed.
The results show that the CVs for negative samples varied extensively, reflecting the fact that a high proportion of the S/CO ratios were close to or below zero. The results show that the majority of S/CO ratio responses observed against the 11 antigens with weak positive samples (63/77) had CVs less than 10%. There were 14 exceptions where the CVs were >10%. Of these, 13/14 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 the majority of S/CO ratio responses with strong positive samples (68/77) had CVs less than 10%. There were 9 exceptions where the CVs were >10%. Of these, 3/9 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%.

Mixed linear models were applied with kit, 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.

Diagnostic reproducibility

An evaluation panel of serum samples comprising negative, weak positive and strong positive serum samples were blinded and analysed 3 laboratories for analytical reproducibility testing in 3 laboratories: APHA Weybridge, UK; Molde Mastitis reference laboratory, NO; and Enfer Scientific routine testing laboratory, IE. Seven negative samples, 7 weak positive samples, and 7 strong positive samples were tested using two plates from two different kit batches and 1 technician in each laboratory. The results from the APHA and Molde laboratories were sent to Enfer Scientific for un-blinding and analysis. The results show complete concordance between the 3 laboratories for binary analysis.

Application

The test has not yet been incorporated into routine diagnostic regimens.

References

Whelan et al (2008). Multiplex Immunoassay for serological diagnosis of *Mycobacterium bovis* infection in cattle. Clin. Vac. Immnunol. 15(12): 1834 – 1838.