World Organisation for Animal Health
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WOAH Procedure for Registration of Diagnostic Kits
Validation Studies Abstract

Name of the diagnostic kit: Enferplex Bovine TB antibody test

Manufacturer: Enfer Scientific ULC

WOAH original approval number: 20190113

New procedure/approval number: 111824

Date of Registration: May 2023

Disease: Bovine tuberculosis

Pathogen Agent: *Mycobacterium bovis*

Type of Assay: Indirect chemiluminescent multiplex ELISA

Purpose of Assay:

Certified by the WOAH as fit for the detection of antibody to *Mycobacterium bovis* in bovine milk samples (May 2023) 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, in particular 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 based on detection of antibodies in individual bovine milk samples excluding colostrum and first milk samples taken within 4 days of calving.

2. As a screening test to identify herds with *Mycobacterium bovis* infection based on detection of antibodies in bovine bulk tank milk samples excluding colostrum and first milk samples taken within 4 days of calving.

Species and Specimens

The test has been validated and approved for testing individual and bulk tank milk samples from cattle.

1. **Information on the kit**

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

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

2. **Summary of validation studies**

Analytical specificity

Individual milk samples

Analytical specificity was assessed using individual milk samples from bovine TB (bTB) free cattle naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* **(**MAP), Bovine Viral Diarrhoea Virus (BVDV), and Infectious Bovine Rhinotracheitis (IBR), Fasciola hepatica FH (FH), Bovine corona virus (BCV) and Bovine Respiratory Syncytial Virus (BRSV). The results are shown in Table 1.

Table 1. Analytical specificity of the Enferplex test using individual milk samples

Sample set	No. samples	Analytical specificity % high sensitivity setting										
		Ag1	Ag2	Ag3	Ag4	Ag5	Ag6	Ag7	Ag8	Ag9	Ag10	Ag11
Map positive	129	99.2	97.7	100	99.2	100	97.7	99.2	99.2	100	97.7	97.7
BVDV positive	611	100	100	100	100	100	100	100	100	100	100	100
IBR gE positive	861	100	100	100	100	100	100	100	100	100	100	100
FH positive	286	99.7	100	99.7	99.7	99.7	100	100	100	100	100	100
BCV positive	536	99.6	100	99.6	99.8	99.8	100	100	100	100	100	100
BRSV positive	1096	99.7	100	99.7	99.8	99.8	100	100	100	100	100	100

The results show very high analytical specificity in individual milk samples from cattle infected with the listed pathogens.

Bulk tank milk samples

Analytical specificity was assessed using bulk tank milk samples from cattle naturally infected with MAP, BVDV, IBR, FH, BCV or BRSV. The results are shown in Table 2.

Table 2. Analytical specificity of the Enferplex test using bulk tank milk samples

Sample set	No. samples	Analytical specificity % high sensitivity setting										
		Ag1	Ag2	Ag3	Ag4	Ag5	Ag6	Ag7	Ag8	Ag9	Ag10	Ag11
MAP positive	148	100	100	99.3	99.3	99.3	100	100	100	100	100	100
BVDV positive	52	100	100	100	100	100	100	100	100	100	100	100
IBR gE positive	1020	100	100	100	100	100	100	100	100	100	100	100
FH positive	158	100	100	100	100	100	100	100	100	100	100	100
BCV positive	1410	99.9	100	99.8	99.9	99.9	99.9	100	100	100	100	100
BRSV positive	1663	99.9	100	99.9	99.9	99.9	99.9	100	100	100	100	99.9

The results show very high analytical specificity in bulk milk samples from herds infected with the listed pathogens.

Conclusion: The specificity of the Enferplex Bovine TB assay was not adversely affected by MAP or other common pathogens of cattle when using individual milk samples or bulk tank milk samples from bTB negative animals.

Analytical sensitivity

Individual and bulk tank milk samples

Analytical sensitivity was estimated for each antigen in the test using endpoint titration of a strong positive individual anamnestic milk sample and a strong positive non-anamnestic bulk milk sample. The results show that the endpoint titres for the individual milk sample ranged from1:160 – 1:2560 across the 11 antigens in the test using individual milk, and 1/20 – 1/2560 using bulk tank milk.

Conclusion. The results show high endpoint titres and dynamic range of the test using individual anamnestic milk samples and good endpoint titres and dynamic range using non-anamnestic bulk milk samples.

Repeatability

Individual milk samples

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

The % CV within-run and between run for weak positive samples ranged from 3.8 – 9.6% and for strong positive samples ranged from 1.4 and 3.9%. The mean values did not exceed 2 SDs over 20 runs of the test.

Bulk tank milk samples

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

The % CV within-run and between-run for weak positive samples ranged from 3.2 – 10.8% and for strong positive samples ranged from 1.4 and 4.0%. The mean values did not exceed 2 SDs over 20 runs of the test.

Conclusion: The Enferplex Bovine Tb antibody test showed very good within well and between run repeatability using individual and bulk tank milk samples.

Diagnostic characteristics

Threshold determination

Thresholds for the individual antigens were set empirically, targeting specificity at 98% for the high sensitivity setting and 99.5% for the high specificity setting of the test. The threshold for overall assay positivity was set based on a 2 – antigen rule, whereby the RLU signals from 2 or more antigens need to be above their individual antigen thresholds for the sample to be registered as “positive”. Sensitivity is maximised by taking the milk sample approximately 5-30 days after a SICCT test. The PPDb injection ‘boosts’ the antibody levels in animals which have been primed through *M. bovis* infection (‘boosted’ sample). If milk is taken outside this timeframe, then no boosting effect would be expected (‘non-boosted’ sample) and the sensitivity is somewhat lower.

Relative diagnostic sensitivity (DSn) and specificity (DSp) estimates

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). Relative diagnostic sensitivity was estimated using boosted individual milk samples from SICCT test positive animals and using non-boosted bulk tank milk samples from SICCT test positive herds in the UK and IE. Diagnostic specificity of individual milk samples was estimated using bTB free animals from the UK, and of bulk tank milk samples using herds from the UK, DK, DE, and NO that were deemed to be free of bTB.

Individual milk samples

Individual boosted milk samples from 305 SICCT test positive animals and from 1149 non-boosted and 195 boosted true negative reference animals from the UK were tested in the Enferplex Bovine TB antibody test. The results are shown in Table 3.

Table 3. Relative sensitivity of the Enferplex Bovine TB antibody test in individual milk samples using the high sensitivity setting

Test method under evaluation	Statistical variable	Target Species – cattle high sensitivity	Target Species – cattle high specificity
Relative diagnostic sensitivity SICCT test positive **Boosted**	N RSn CI	**305** **90.8%** **87.1-93.6**	**305** **87.2%** **83.0-90.6**
Relative sensitivity SICCT positive, bTB lesion positive Boosted	N RSn CI	**83** **95.2%** **88.3-98.1**	**83** **90.4%** **82.1-95.0**
Relative diagnostic specificity SICCT test negative and/or OTF status and Bovine TB history **Non-boosted**	N RSp CI	**1149** **99.7%** **99.2-99.9**	**1149** **99.8%** **99.4-100.0**
Relative diagnostic specificity SICCT test negative and/or OTF status and Bovine TB history **Boosted**	N RSp CI	**195** **98.5%** **95.6-99.5**	**195** **99.5%** **97.2-99.9**

The results show that the relative sensitivity was 90.8% and 87.2% using the high sensitivity and high specificity settings of the test respectively in boosted individual milk samples from SICCT test positive herds. In SICCT positive, lesion positive animals, the relative sensitivity was 95.2% and 90.5% using the high sensitivity and high specificity settings of the test respectively. The specificity was 99.7% using the high sensitivity setting and 99.8% using the high specificity setting of the test in bTB free herds. The relative specificity in boosted individual milk samples from bTB-free animals was 98.5% and 99.4% using the high sensitivity and high specificity settings of the test respectively.

Kappa agreement analysis between the Enferplex test and SICCT test results gave a Kappa value of 0.934 95% CI: (0.911-0.957) showing almost perfect agreement using boosted individual milk samples. Similarly, a Kappa value of 0.951 (95% CI: 0.911-0.973) was found between the Enferplex test and SICCT positive, lesion positive animals, indicating almost perfect agreement. Almost perfect agreement was observed using Kappa analysis between Enferplex antibody results and SICCT test status in boosted samples from SICCT test positive animals and boosted samples from bTB negative animals.

Likelihood ratio (LR) analysis was performed taking test outputs with a $LR^+ > 10$ or $LR^- < 0.1$ as good diagnostic evidence of the infection being either present or absent respectively (Caraguel & Colling, 2021). The Likelihood ratio (LR) for positive LR+ and LR^- were 347.8 (95% CI: 112.3-1077.5) and 0.092 (95% CI: 0.065-0.131) respectively for boosted samples from SICCT positive animals. The diagnostic odds ratio (DOR) was 3779.1 In boosted samples from SICCT positive animals with lesions, the LR+ and LR- were 364.5 (95% CI: 117.6 – 1129.8) and 0.048 (95% CI: 0.019-0.126) respectively. The DOR was 7544.5.

Analysis of paired milk and serum samples from 199 boosted SICCT test positive animals using Spearman's Rank correlation test gave coefficients ranging between 0.78 – 0.96 for the individual antigens used in the Enferplex test. The results thus showed good correlation between serum and milk samples. Analysis of paired serum and milk results using the McNemar discrimination test showed that the differences in proportions between serum and milk were not statistically significant at either the high sensitivity setting or the high specificity setting of the test. Similar high correlations between

serum and milk sample results were obtained when the number of antigens recognised by antibody was used instead of continuous data.

Conclusion: The results indicate that individual milk samples could be used instead of serum for the serodiagnosis of bTB using the Enferplex Bovine TB antibody test.

Bulk tank milk samples.

The relative diagnostic sensitivity and specificity of the Enferplex Bovine TB antibody test was estimated using bulk tank milk samples from bTB breakdown herds and bTB-free herds respectively.

Bulk tank milk samples from 235 SICCT positive herds and from 1792 true negative reference herds in the UK and Europe were tested in the Enferplex Bovine TB antibody test. The bulk tank milk samples from bTB positive herds were taken at the time of reading the SICCT test and were therefore non-boosted. The results are shown in Table 4.

Table 4. Relative sensitivity and specificity estimate of the Enferplex Bovine TB antibody test using non-boosted bulk tank milk samples

Test method under evaluation	Statistical variable	Target Species – cattle High Sensitivity	Target Species – cattle High Specificity
Relative diagnostic sensitivity SICCT positive	N RSn CI	**247** **77.7%** **72.1-82.5**	**247** **71.7%** **65.4-76.9**
Relative diagnostic specificity SICCT negative and/or OTF status and Bovine TB history	N RSp CI	**1792** **99.8%** **99.4-99.9**	**1792** **99.9%** **99.6-99.9**

The results show that the relative sensitivity was 77.7% and 71.7% using the high sensitivity and high specificity settings of the test respectively in non-boosted bulk tank milk samples from SICCT test positive herds. The specificity was 99.8% using the high sensitivity setting and 99.9% using the high specificity setting of the test in bTB free herds. Bulk tank milk samples were grouped depending on country of origin and the specificity obtained in the Enferplex Bovine TB antibody test compared. The results show that the specificity ranged between 99.0 – 100%, indicating that the diagnostic specificity of the Enferplex Bovine TB antibody test did not differ significantly between countries.

The relative sensitivity for bulk tank milk samples with low SICCT test prevalence (0.1 – 1.0%) was 74.1% using the high sensitivity setting of the test. No significant differences were noted in the Enferplex test relative sensitivity in relation to reactor prevalence, herd size or milk yield. Kappa agreement analysis between the Enferplex test bulk tank milk results and SICCT test results gave a Kappa value of 0.842 showing almost perfect agreement.

The likelihood ratio (LR) for positive (LR^+) and negative (LR^-) bulk tank milk samples were 348.0 and 0.223 respectively. The DOR was 1560. Test outputs with an $LR^+ > 10$ or $LR^- < 0.1$ are considered good diagnostic evidence of the infection being either present or absent respectively.

Conclusion: The results show that the Enferplex Bovine TB antibody test can be used to confirm the results of the SICCT test and as a screening test for bTB using non-boosted bulk tank milk samples.

Reproducibility

Evaluation panels of samples comprising negative, weak positive and strong positive individual milk and bulk tank milk samples were blinded and sent to the 3 independent 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.

A series of linear mixed effect models were run with kit batch, laboratory, and sample considered. The results included the overall means, SD, CV, the 95% CI with the Upper and Lower Control Limits, an estimate of how much variation was due to these variables, and statistical analysis of the differences observed.

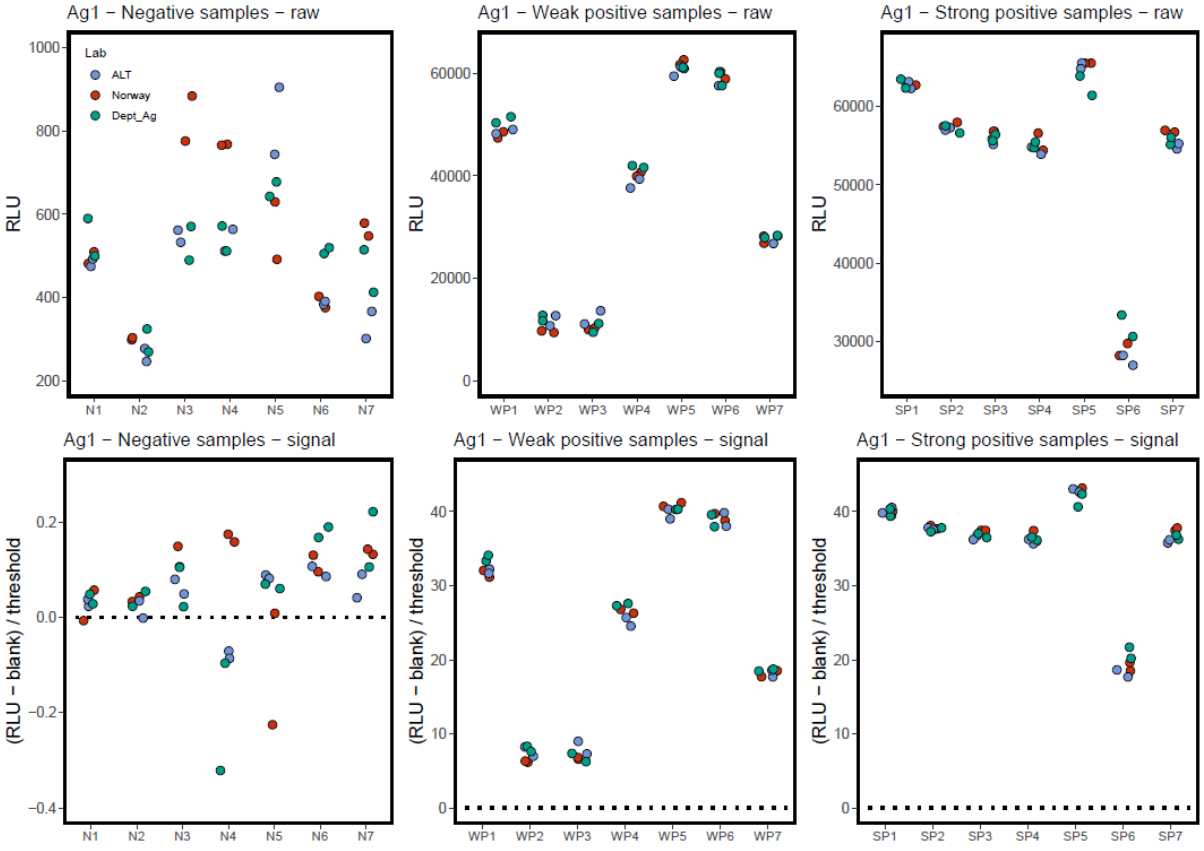
Analytical reproducibility

Individual milk samples

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 showed that most of the S/CO ratio responses with WP and SP samples had CVs less than 10%. There were 31 results where the CVs were >10%. Of these, 23/31 were associated with responses that were below threshold for the individual antigens and would be deemed to be negative responses for those antigens. The CVs of the remaining 9 were 10.4, 10.4, 11.2, 12.0, 12.1, 12.4, 12.6, 13.3, and 15.9%. Analyses using mixed linear models showed that for the WP and SP samples, 98 – 100% of the variation was due to the sample and none was due to the kit or laboratory.

An example of the individual milk reproducibility RLU and S/CO data for Ag 1 obtained by three laboratories (colour coded in duplicate) is shown in Figure 1. The cut-off for S/CO ratio is 1.

Figure 1. Individual milk reproducibility data for Ag1.



Conclusion: The Enferplex Bovine TB antibody test thus shows good analytical reproducibility between kits and laboratories when testing individual milk samples.

Diagnostic reproducibility

The diagnostic reproducibility results for individual milk samples are shown in Table 5.

Table 5. Summary of diagnostic reproducibility testing using the 2 Ag rule

Samples	Number positive/tested		
	Laboratory 1	Laboratory 2	Laboratory 3
Positive control	2/2	2/2	2/2
Negative control	0/2	0/2	0/2
Blinded Negatives	0/7	0/7	0/7
Blinded weak positives	7/7	7/7	7/7
Blinded strong positives	7/7	7/7	7/7
Blinded weak positives	7/7	7/7	7/7
Blinded strong positives	7/7	7/7	7/7

The results show complete concordance between the 3 laboratories. The results demonstrate high reproducibility of the Enferplex Bovine TB antibody test when used in 3 different laboratories with 2 different kit batches using individual milk samples.

Analytical reproducibility

Bulk tank milk

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 showed that most of the S/CO ratios above threshold with WP and SP bulk milk samples had CVs less than 10%. Higher CVs were associated with samples below mean value threshold. There were 17 results where the CV% was >10%. Of these, only 2/17 were above 20% (20.8%; 26.8%).

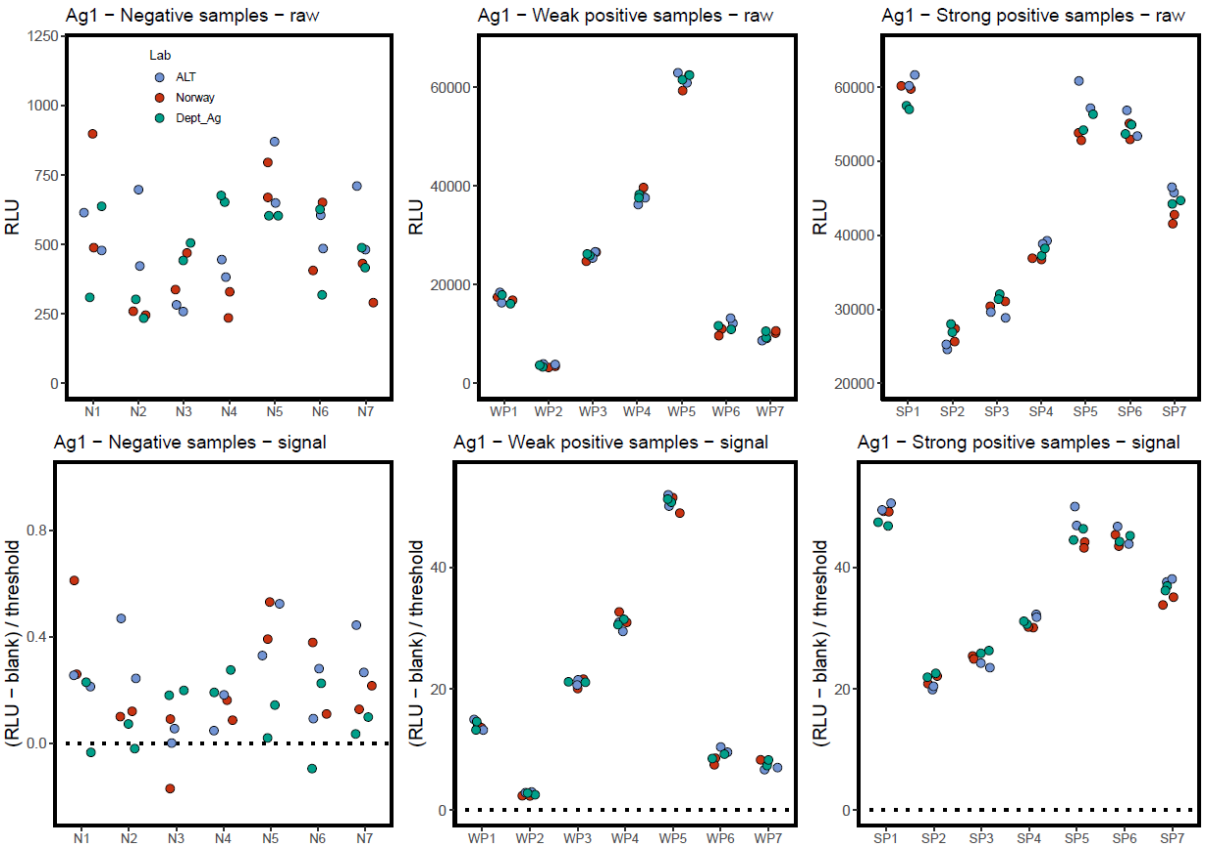
Analysis using mixed linear models showed that for the WP and SP samples, 85 – 100% of the variation was due to the sample and none was due to the kit or laboratory. The Enferplex Bovine TB antibody test thus shows good reproducibility between kits and laboratories using non-boosted bulk tank milk samples.

Diagnostic reproducibility

Bulk tank milk

Bulk tank milk diagnostic reproducibility was assessed in three independent laboratories and the results sent to Enfer Scientific for un-blinding and analysis. The results showed complete concordance between the 3 laboratories using different 2 kits. Representative plots of the raw RLU values and S/CO ratios obtained for Ag1 using negative, weak positive, and strong positive bulk tank milk samples are shown in Figure 2. The duplicate values from each lab for each sample colour coded for laboratory are shown. The cut-off for S/CO ratio is 1.

Figure 2. Bulk tank milk reproducibility for Ag 1.



Conclusion: The Enferplex Bovine TB antibody test thus shows good analytical reproducibility between kits and laboratories when testing bulk tank milk samples.

The diagnostic reproducibility results for bulk tank milk samples are shown in Table 6.

Table 6. Summary of diagnostic reproducibility testing using the 2 Ag rule

Samples	Number positive/tested		
	Laboratory 1	Laboratory 2	Laboratory 3
Positive control	2/2	2/2	2/2
Negative control	0/2	0/2	0/2
Blinded Negatives	0/7	0/7	0/7
Blinded weak positives	7/7	7/7	7/7
Blinded strong positives	7/7	7/7	7/7
Blinded weak positives	7/7	7/7	7/7
Blinded strong positives	7/7	7\7	7/7

The results show complete concordance between the 3 laboratories. The results demonstrate high reproducibility of the Enferplex Bovine TB antibody test when used in 3 different laboratories with 2 different kit batches using bulk tank milk samples.

Reference

Caraguel, C.G.B. & Colling A. (2021). Diagnostic likelihood ratio – the next generation of diagnostic test accuracy measurement. Rev. Sci. Tech. Off. Int. Epiz. 40(1): 299-309.