

Application of Enzyme-Linked Immunosorbent Assay (ELISA) Antibody Test and Test Strips in Detection of Bovine Tuberculosis

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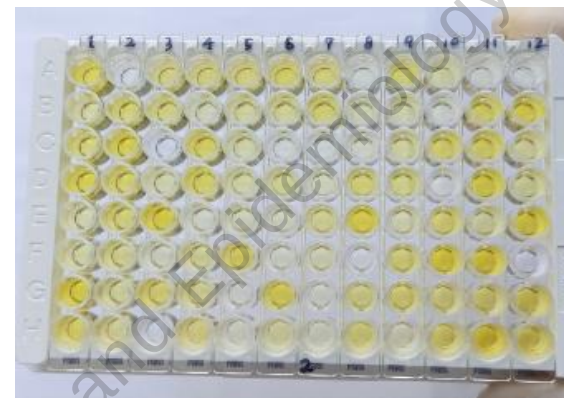
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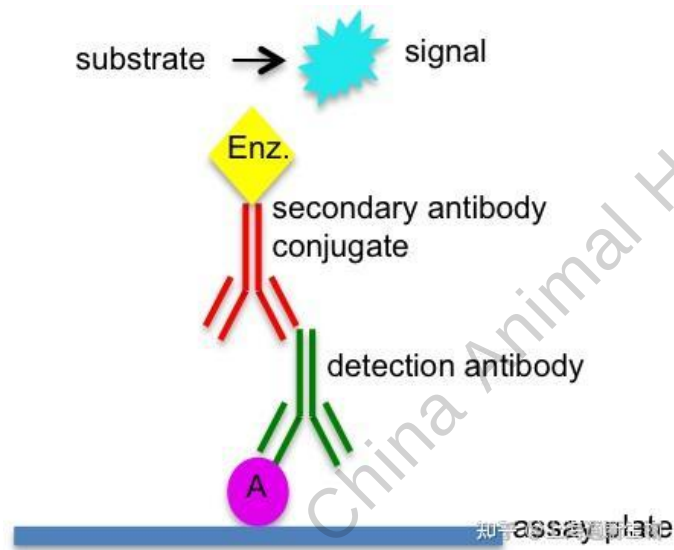
**WOAH Regional Hands-on Training on
Zoonotic TB Diagnosis**

**Qingdao, China P.R., 14 – 18 September
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- ELISA is a comprehensive technology that combines the immune reactions of antigens and antibodies with the efficient catalytic reactions of enzymes.
- According to the binding mode of antigen antibody reaction, there are direct method, indirect method, sandwich method, competitive method.
- For bovine TB detection, **indirect method (iELISA)** is the most widely used ELISA method.





Indirect ELISA uses two types of antibodies: the first antibody (primary antibody) specifically recognizes the test antigen, and the second antibody (secondary antibody) specifically recognizes the primary antibody.

Firstly, the test antigen is adsorbed into the wells of the reaction plate and washed to remove unbound antigen.

Then, add the primary antibody, which binds to the antigen.

Next, enzyme labeled secondary antibodies are added, which bind to the primary antibody.

Finally, the substrate solution is added, and the enzyme catalyzes a color change in the substrate. The degree of color change is proportional to the amount of antigen in the sample.



1. Preparation

We need to label every column of the ELISA plate clearly to prevent them from falling off during slapping, which could make the samples disordered.

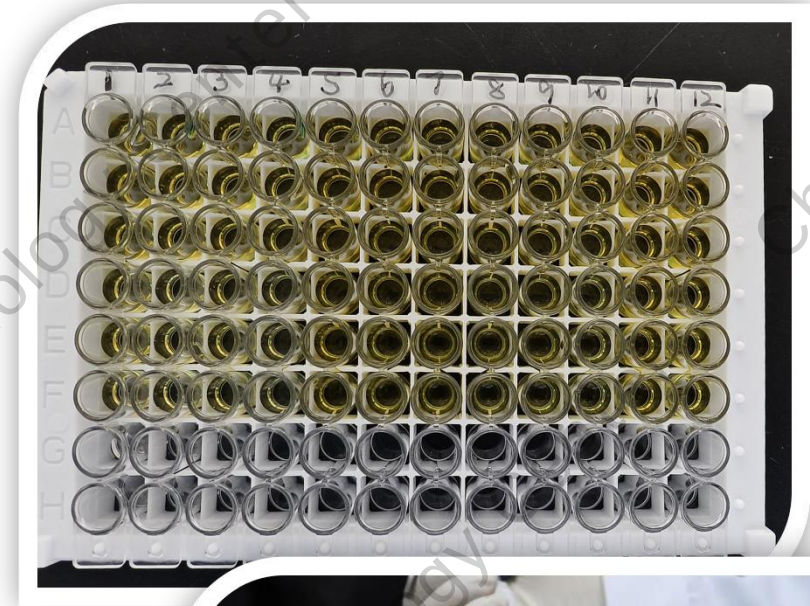
2. Washing

After washing the plate, we need to slap the plate on the table hard, preventing liquid residue in the plate and affect the experimental results.

When washing the plate, remember to tap the four corners of the plate to ensure washing thoroughly.

3. Adding samples

When adding samples, pay attention to operate gently to avoid the liquid splashing into adjacent sample wells, resulting some inaccurate experimental results.





4. About TMB Color liquid

When adding TMB Color liquid, do not expose the TMB Color liquid to illumination for too long time, avoiding the failure of the liquid.

5. About the stopping solution

When adding the stopping solution, there is no need to pour out the TMB Color liquid or wash the plate, just add it directly.

6. About the Microplate reader

After termination of the color reaction, the plate needs to be placed in the machine immediately, so the Microplate reader needs to be turned on in advance.





Whole *M. bovis* Antigen

In 1987, Ritacco *et al.* developed the first serological test to detect antibodies in *M. bovis*-infected cattle. In their study, an ELISA based on PPDB yielded 90% sensitivity and 89.9% specificity when testing serum samples from confirmed *M. bovis*-infected and healthy cattle. Subsequent analysis with a larger number of animals showed lower sensitivity (73.6%) but higher specificity (94.1%).

Another study using whole-cell sonicate as an antigen in an ELISA test showed lower specificity (52.6%) and sensitivity (63%—88.7%).

The poorer performance of the test with the whole-cell sonicate could be due to cross-reactions with environmental or nontuberculous mycobacteria or to the absence of *M. bovis*-secreted proteins.



Individual Antigens

The proteins **MPB70 and MPB83** have been the most extensively studied and are now commonly used as antigens for serological tests in bTB because of their immunogenic characteristics. Animals infected or sensitised with *M. avium* have shown low or null reactions to MPB70/MPB83. These findings indicate reliable performance of MPB70/MPB83 as *M. bovis*-specific antigens.

Ag85A is a member of the A85 complex, which includes Ag85B and Ag85C. These proteins are major secreted proteins of *M. tuberculosis* and *M. bovis*. Two studies have reported acceptable sensitivity (54.2%) and specificity (83.5%) for Ag85A when used in a competitive ELISA.

ESAT6 and CFP10 are potent T cell antigens secreted by *M. tuberculosis* and *M. bovis*. These antigens are highly recognized by T cells from infected individuals, making them significant markers for the immune response. Fusion proteins containing peptides from ESAT6 and CFP10 have also shown good sensitivity and specificity when evaluated in TST-positive cattle (sensitivity: 69.4–83.2%, specificity: 86.5–96.0%).

1. The Enferplex TB method is an assay developed by EnferGroup that incorporates 11 antigens and is currently the only commercially available multiplexed assay to detect bTB in cattle.

2. Another serological test commercially available for TB is the IDEXX ELISA, which is an ELISA in a 96-well microtiter plate format that detects antibodies to two *M. tuberculosis* complex antigens (MPB70 and MPB83).

3. **Enferplex and IDEXX serological kits** can be used as ancillary methods to cell-mediated diagnostic tests in chronically infected herds. Additionally, **both tests showed higher sensitivity after the application of PPDB**, with Enferplex apparently having better performance.

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

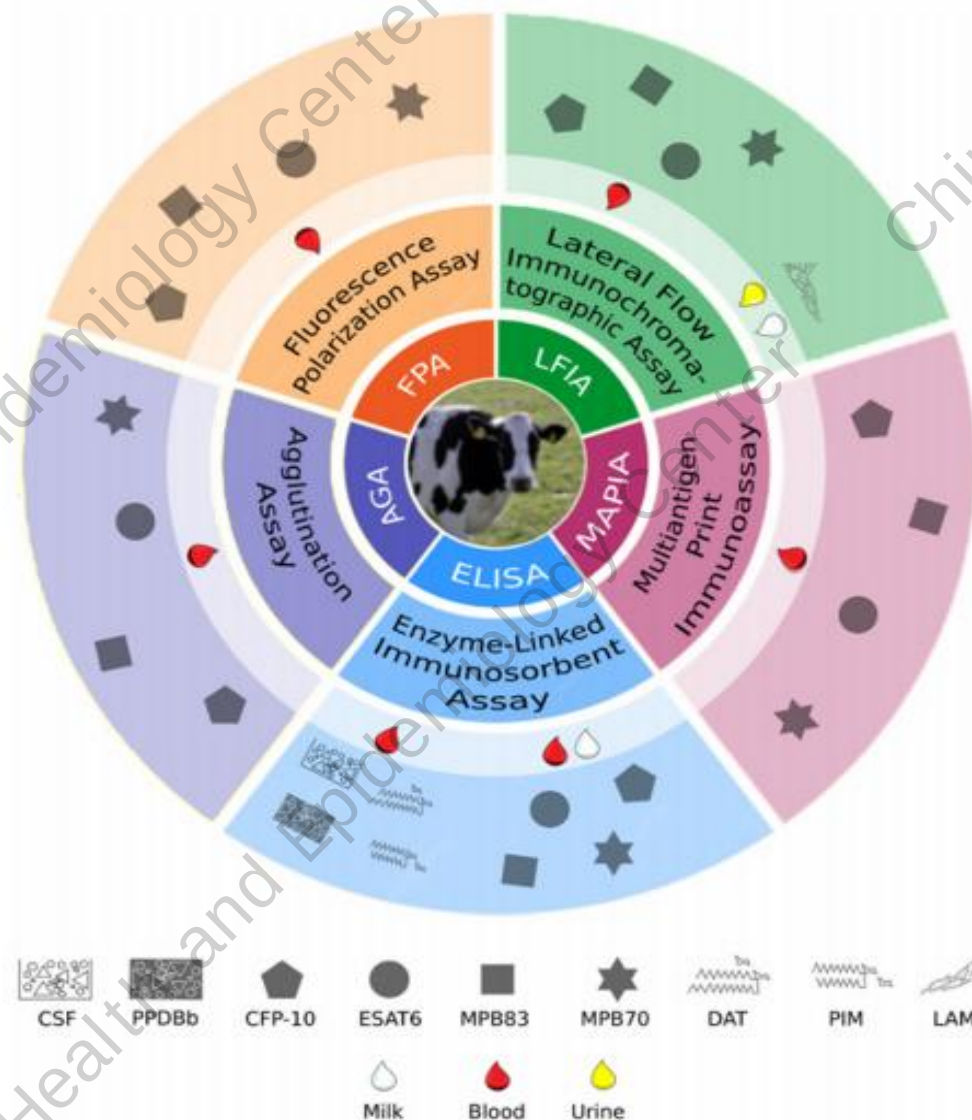
Amanda O'Brien¹, John Clarke¹, Alastair Hayton², Andy Adler², Keith Cutler², Darren J. Shaw³, Clare Whelan¹, Neil J. Watt⁴ & Gordon D. Harkiss^{4,5}

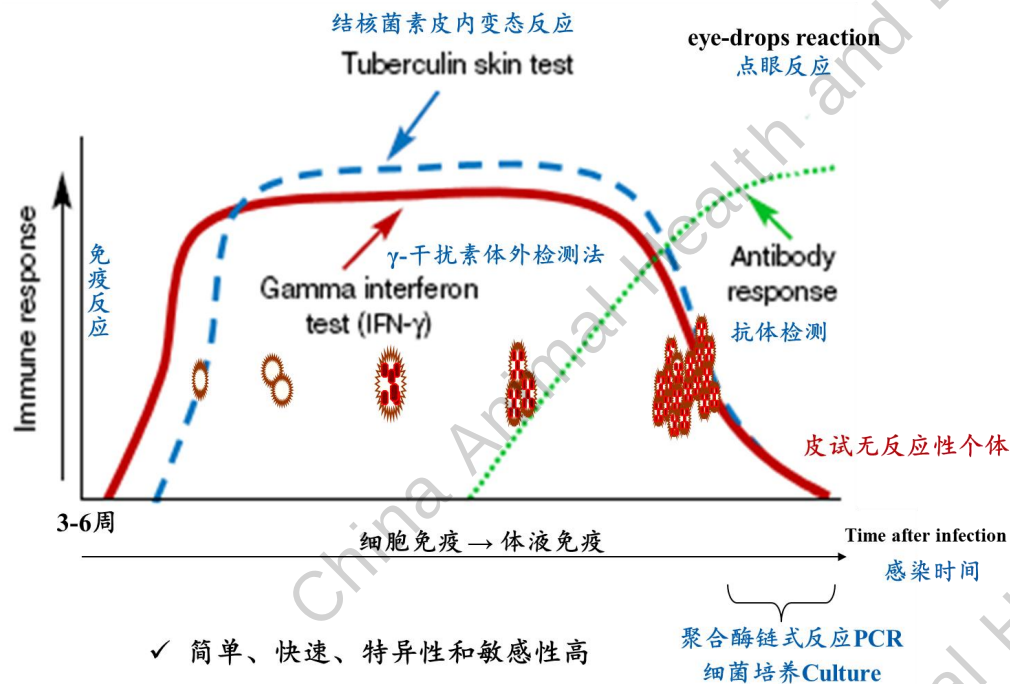
Bovine tuberculosis is a contagious bacterial disease of worldwide economic, zoonotic and welfare importance caused mainly by *Mycobacterium bovis* infection. Current regulatory diagnostic methods lack sensitivity and require improvement. We have developed a multiplex serological test for bovine tuberculosis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



ELISA is predominantly used for the serological diagnosis of bTB by using different antigens such as MPB70, MPB83, ESAT6, CFP10, PPDB, and total proteins from cellular and culture supernatants of *M. bovis*. The sensitivity and specificity of ELISA vary depending on the antigens used, the immunoglobulins detected, and the type of biological sample (e.g., blood, urine, milk). Generally, the specificity is over 90%, while the sensitivity ranges from less than 20% to 100%.

Key platforms for antibody detection in tuberculous animals.





WOAH: ELISA antibody detection method is the only serological method that can be used as an auxiliary means rather than a substitute for bovine tuberculosis detection at present.

We need to consider using ELISA in the following conditions:

1. Widespread infection in cattle herds.
2. Cattle with severe disease progression.
3. Cattle with negative skin test and IFN - γ test results in the late stage of bovine tuberculosis infection.

Worthy of remark:

1. At the early stage of bovine tuberculosis infection, ELISA method is less sensitive than skin test and IFN - γ in vitro release method.
2. ELISA is relatively simple to operate, but not suitable for large-scale screening.



- **Fluorescent microsphere antibody test strips**
- **IFN- γ detection method based on colloidal gold test strips**



Establishment of Fluorescent Microsphere Antibody Test Strips For Convenient Diagnosis of Bovine Tuberculosis

1. Expression plasmid construction

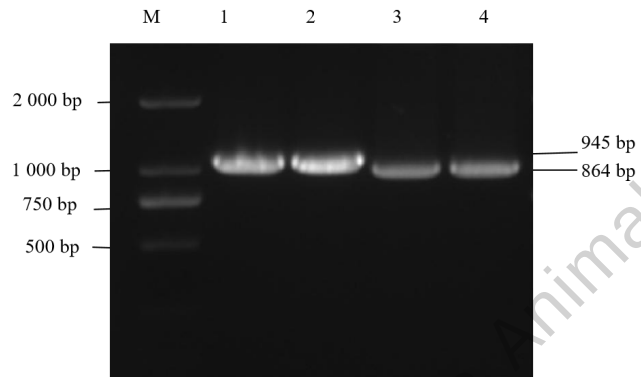


Fig. 1 Identifying plasmids through agarose gel electrophoresis

2. Expression and purification of recombinant MPB70 and MPB83 proteins

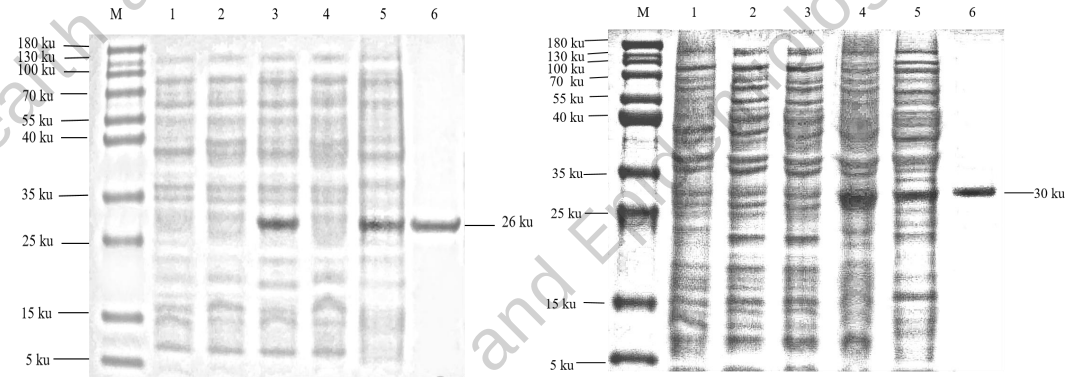


Fig. 2 Identifying the MPB70 and MPB83 proteins through SDS-PAGE

3. Western Blot

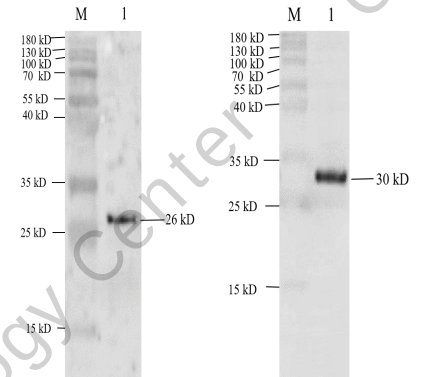


Fig. 3 Validation of the immunogenicity of recombinant MPB70 and MPB83 proteins

Table 1 Screening of optimal protein-to-fluorescent bead ratio

Serum		Different protein-to-fluorescent bead ratios		
		2:1	1:1	1:2
Positive serum	T	956 314	865 444	3 348 420
	C	5 033 231	4 973 839	4 973 886
	T/C	0.191	0.174	0.673
Negative serum	T	348 491	291 819	1 243 174
	C	4 840 152	4 912 777	4 835 371
	T/C	0.072 1	0.059 4	0.257
P/N		2.64	2.92	2.62

Set the mass ratio of **MPB83** protein to microspheres to 2:1, 1:1, or 1:2.

Screening based on the P/N value revealed that a mass ratio of **1:1** represents the optimal coupling ratio.

Table 2 Screening of optimal coating concentration for T line

Serum		Different coating concentrations for T line			
		0.1	0.2	0.3	0.4
		mg/mL	mg/mL	mg/mL	mg/mL
Positive serum	T	786 680	1 455 481	1 070 774	1 015 530
	C	4 898 387	4 932 163	4 927 632	4 908 314
	T/C	0.161	0.295	0.218	0.207
Negative serum	T	303 081	489 320	376 698	388 886
	C	4 936 174	4 891 734	4 911 326	4 922 613
	T/C	0.061 4	0.101	0.076 7	0.079
P/N		2.61	2.94	2.83	2.61

Table 3 Screening of optimal coating concentration for C line

Serum		Different coating concentrations for C line			
		0.2	0.3	0.4	0.5
		mg/mL	mg/mL	mg/mL	mg/mL
Positive serum	T	1 145 226	1 228 712	1 446 483	1 471 613
	C	4 276 425	4 512 348	4 941 861	5 326 143
	T/C	0.268	0.272	0.293	0.276
Negative serum	T	474 143	464 509	487 963	536 052
	C	4 306 479	4 492 356	4 889 408	5 307 454
	T/C	0.111	0.103	0.099 8	0.101
P/N		2.43	2.63	2.93	2.73

The optimal coating mass concentration for the **MPB70** protein for Line T is **0.2 mg/mL**.

The optimal coating mass concentration for the goat anti-chicken antibody for Line C is **0.4 mg/mL**.

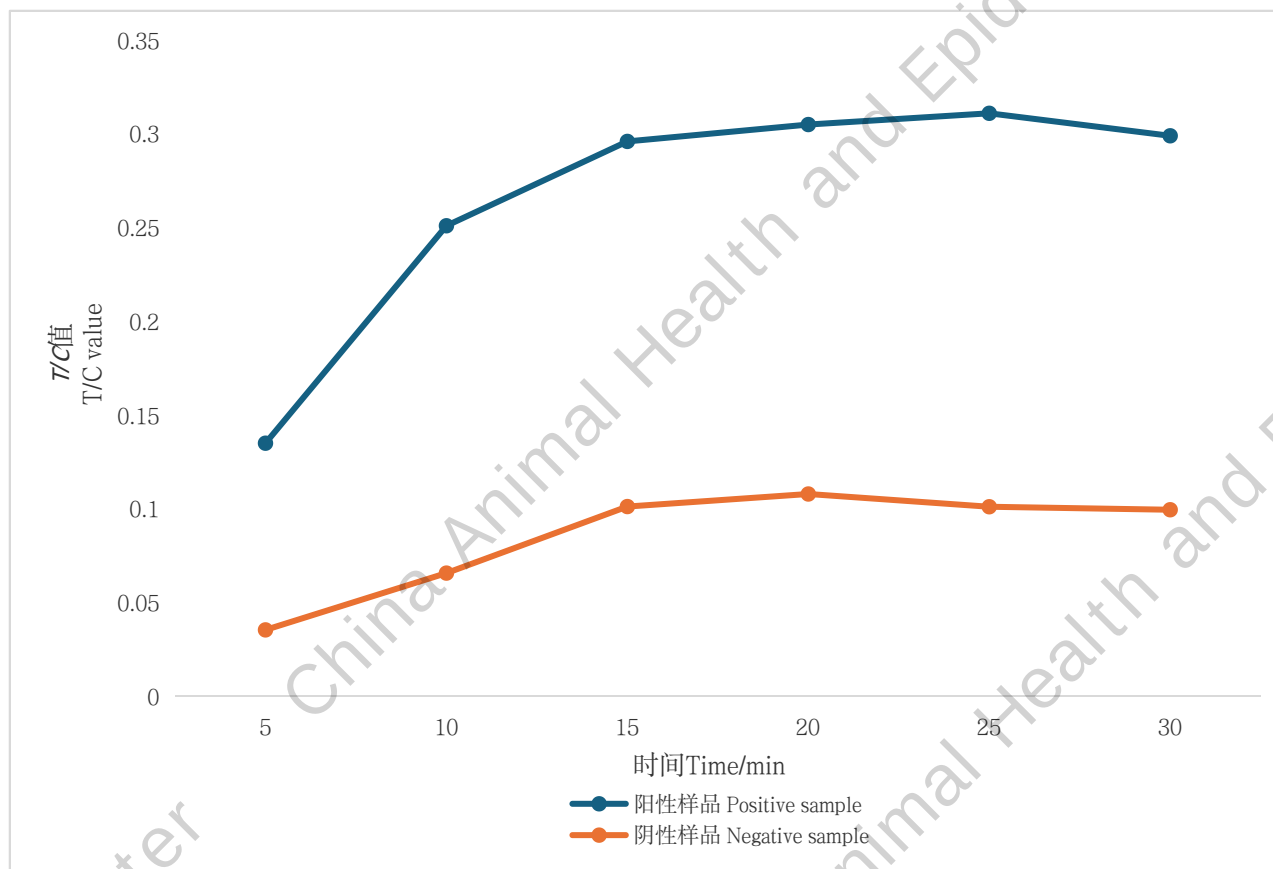


Fig. 4 Changes in the T/C values of Bovine tuberculosis antibody fluorescent microsphere test strips over time

The T/C levels in both positive and negative serum samples at various time points were calculated.

It was observed that these levels stabilized after **15 minutes** and remained constant throughout the detection period.

Therefore, it was determined that T/C measurements should commence **15 minutes** after the reaction began.



Table 4 Screening of optimal serum dilutions

Serum		Different serum dilutions					
		5	10	25	50	100	200
Positive serum	T	1 985 014	4 874 520	2 619 828	1 457 661	1 061 044	968 321
	C	4 861 658	4 874 526	4 893 217	4 939 451	4 914 517	4 927 846
	T/C	0.408	0.754	0.535	0.295	0.216	0.197
Negative serum	T	467 177	512 275	496 712	495 784	475 545	500 540
	C	4 938 456	4 911 554	4 836 542	4 894 217	4 862 431	4 902 451
	T/C	0.094 6	0.104	0.103	0.101	0.097 8	0.102
P/N		4.32	7.23	5.21	2.91	2.21	1.93

The serum samples with positive and negative bovine tuberculosis antibodies were diluted by 5, 10, 25, 50, 100 and 200 times, respectively.

The results showed that the P/N value was maximized when the serum dilution ratio was **1:10**.



Table 5 Detection results of negative samples

Serum ID	T/C	Serum ID	T/C	Serum ID	T/C
1	0.2270	18	0.1370	35	0.2260
2	0.5510	19	0.2310	36	0.1910
3	0.1510	20	0.4690	37	0.0733
4	0.4910	21	0.1540	38	0.2090
5	0.2970	22	0.2380	39	0.1920
6	0.1150	23	0.0614	40	0.1050
7	0.0967	24	0.0656	41	0.2030
8	0.2940	25	0.3710	42	0.2006
9	0.2070	26	0.0541	43	0.1680
10	0.1660	27	0.0908	44	0.1580
11	0.2440	28	0.1090	45	0.2520
12	0.1950	29	0.2080	46	0.1210
13	0.3740	30	0.1740	47	0.0776
14	0.4440	31	0.2590	48	0.0948
15	0.1990	32	0.0647	49	0.1060
16	0.2850	33	0.0917	50	0.1250
17	0.3520	34	0.1730		

Based on the average value of T/C for negative samples (0.202) plus 3 times the standard deviation (0.116), the positive/negative decision threshold for this method is determined to be **0.55**.



Table 6 Results of the specificity test

Specificity control samples	T/C	Result
Foot-and-mouth disease virus positive serum	0.246±0.109	negative
Bovine herpesvirus positive serum	0.331±0.056	negative
Bovine viral diarrhea mucosal positive serum	0.184±0.019	negative
Mycobacterium paratuberculosis positive serum	0.452±0.102	negative
Infectious bovine rhinotracheitis positive serum	0.203±0.097	negative
Bovine parainfluenza virus 3 positive serum	0.195±0.053	negative
Brucellosis positive serum	0.271±0.084	negative

The detection results using the bovine tuberculosis antibody fluorescent microsphere assay on seven positive serum samples revealed that the serum samples testing positive for FMDV, BROV, BVDV, MAP, IBRV, BPIV3, or Brucella were all **negative**, demonstrating **high specificity**.

Table 7 Results of the sensitivity test

Serum dilution	T/C		
	1	2	3
1:2	15.171	14.564	15.621
1:4	6.909	7.325	6.5421
1:8	4.117	3.861	4.245
1:16	2.301	2.115	2.542
1:32	1.247	1.451	1.364
1:64	0.629	0.648	0.676
1:128	0.361	0.374	0.369

Using positive serum with an antibody titer of 1:64 (as provided in the IDEXX Tuberculosis Antibody Detection Kit) as the test serum, and noting that the maximum dilution at which the test result was positive was **1:64**, this indicates that **the sensitivity of this method is equivalent to that of the IDEXX kit**.



Table 8 Results of repeatability test

Serum number	T/C	Percent coefficient of variation (% CV)
Positive serum 1	15.119±0.530	3.51
Positive serum 2	6.925±0.392	5.66
Positive serum 3	4.074±0.196	4.81
Positive serum 4	2.319±0.214	9.23
Positive serum 5	1.354±0.102	7.56
Negative serum 1	0.281±0.0148	5.29
Negative serum 2	0.119±0.059	5.01
Negative serum 3	0.0998±0.041	4.13
Negative serum 4	0.307±0.016	5.33
Negative serum 5	0.224±0.064	8.53

The coefficient of variation ranges from **3.51% to 9.23%**, indicating that this method exhibits **good repeatability**.

Table 9 Coincidence rate test results

Category	The detection results of the IDEXX bovine tuberculosis antibody test kit in the united states		Total
	+	—	
The detection results of bovine tuberculosis using the fluorescent-microsphere method	23	13	36
	4	139	143

This method was used in conjunction with the IDEXX Bovine Tuberculosis Antibody Detection Kit to simultaneously test **179** clinical bovine serum samples positive for γ -interferon; the overall concordance rate was **90.5%**.



Establishment of Bovine Tuberculosis IFN- γ Detection Method Based on Colloidal Gold Test Strips



Coating Ab	Detection Ab (OD _{450nm} Value)				
	3H11C1A7	4F9G1	3H11C1G7	4F9F2C10	4F9G2E2
3H11C1A7	0.0395	1.9247	1.6134	1.9432	2.1449
4F9G1	1.8801	0.4426	1.7604	1.7617	1.0231
3H11C1G7	1.4191	1.9789	0.0349	2.2288	2.1463
4F9F2C10	1.8582	0.8187	1.8208	0.0471	0.869
4F9G2E2	1.78	1.624	1.8272	1.674	0.0423

- Good antibody pairing performance was observed when 3H11C1A7 and 3H11C1G7 were used as capture antibodies, with 4F9G1, 4F9F2C10 and 4F9G2E2 serving as detection antibodies.
- The pairing of 3H11C1G7 as the capture antibody and 4F9F2C10 as the detection antibody yielded the highest readout when bovine plasma was used as the antigen, representing the optimal antibody combination.

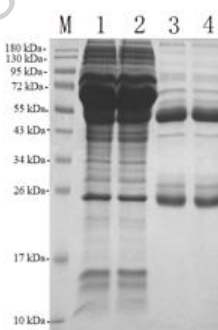
	4F9G1			4F9G2E2			4F9F2C10		
	Negative plasma	Positive plasma	Diluent	Negative plasma	Positive plasma	Diluent	Negative plasma	Positive plasma	Diluent
3H11C1A7	0.2237	1.8866	0.226	0.1993	1.9426	0.1835	0.1392	1.7512	0.1142
3H11C1G7	0.1802	1.8505	0.1614	1.1635	1.9789	0.1508	0.172	2.1766	0.163

hybridoma cell	OD ₄₅₀ value
3H11C1A7	3.0789
4F9G1	2.9497
3H11C1G7	3.0229
4F9F2C10	3.0984
4F9G2E2	2.9497

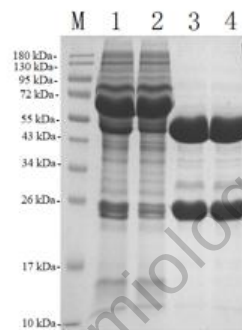
- Five positive cell lines with **high readings** were screened by ELISA. The selected cell lines were inoculated into mice, and ascites was collected and purified to prepare monoclonal antibodies.



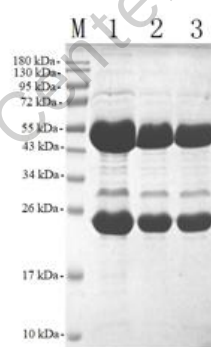
(a) 3H11C1G7



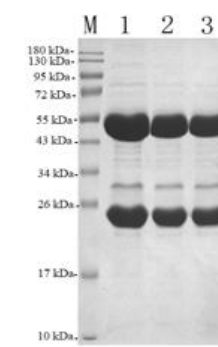
(b) F9G1



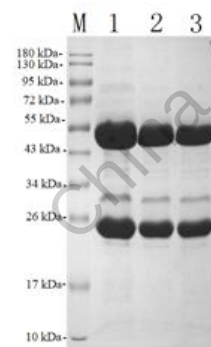
(c) 3H11CIA7



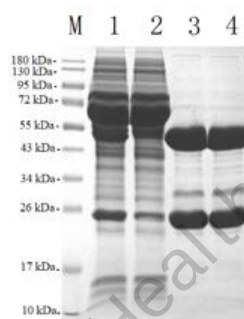
(a) 3H11C1G7



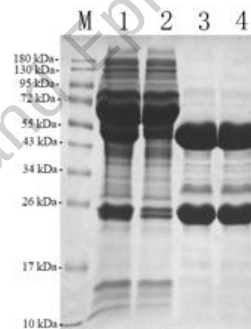
(b) F9G1



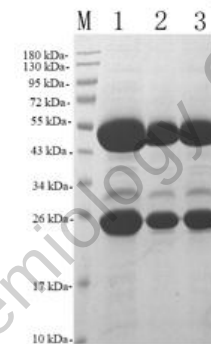
(c) 3H11CIA7



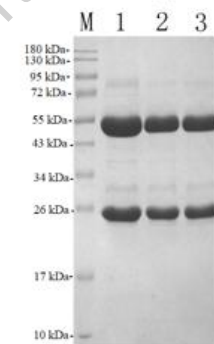
(d) 4F9G2E2



(e) 4F9F2C10



(d) 4F9G2E2



(e) 4F9F2C10

- 1: Loading sample
- 2: Flow-through
- 3: Neutralized eluate
- 4: Filtered eluate

- 1: Pre-exchange liquid
- 2: Post-exchange liquid
- 3: Filtered post-exchange liquid



Preliminary screening: Colloidal gold was adjusted to pH 4, 5, 6, 7, 8 and 9. 400 μ L aliquots were mixed with monoclonal antibodies, and the pH yielding the reddest color was recorded.

pH4.0	pH5.0	pH6.0	pH7.0	pH8.0	pH9.0
Purple	Pale purple	Red	Red	Red	Red



- Bovine IFN- γ mAbs exhibited the reddest color at **pH 6.0 and 7.0**, achieving good colloidal gold labeling.

Rescreening: Colloidal gold was adjusted to pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. 400 μ L aliquots were mixed with monoclonal antibodies, and the pH yielding the reddest color was recorded.

pH5.5	pH6.0	pH6.5	pH7.0	pH7.5	pH8.0	pH8.5	pH9.0
Pale purple	Red	Red	Red	Red	Red	Red	Red



- Bovine IFN- γ mAbs exhibited the reddest color at **pH 6.5, 7.0 and 7.5**, achieving favorable colloidal gold labeling.

The capture antibody was striped onto NC membrane (T line, C line), dried at 37 °C for 1 h, and then laminated onto the PVC backing card.



The conjugate pad was immersed in conjugate pad treatment buffer for over 20 min. taken out, dried at 37 °C for more than 2 h, and laminated onto the PVC backing card.



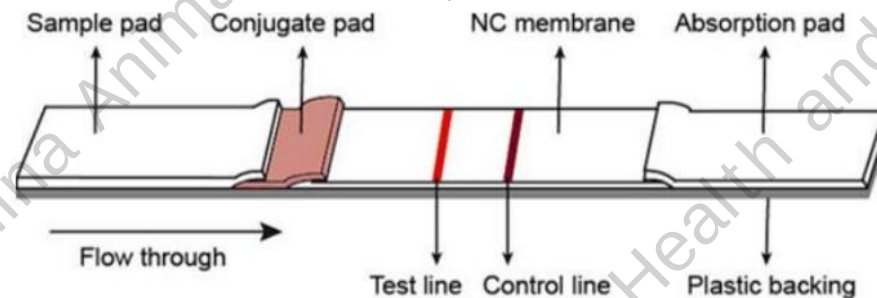
The sample pad was laminated onto the PVC backing card.

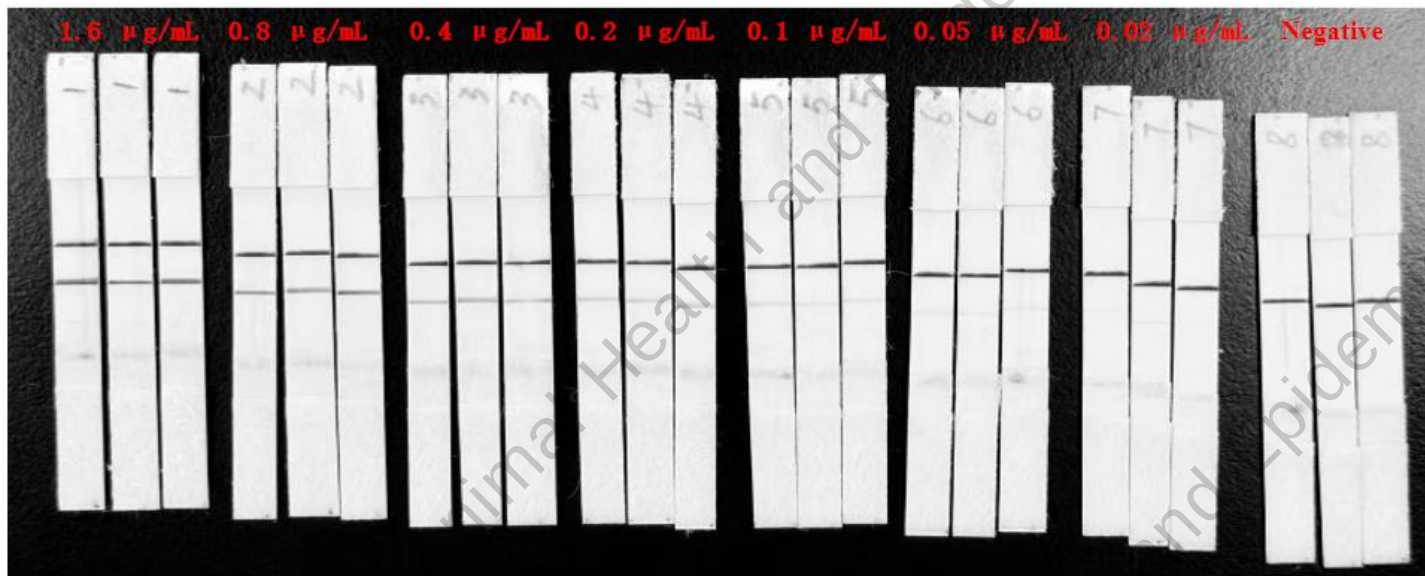


The absorbent pad was laminated onto the PVC backing card.

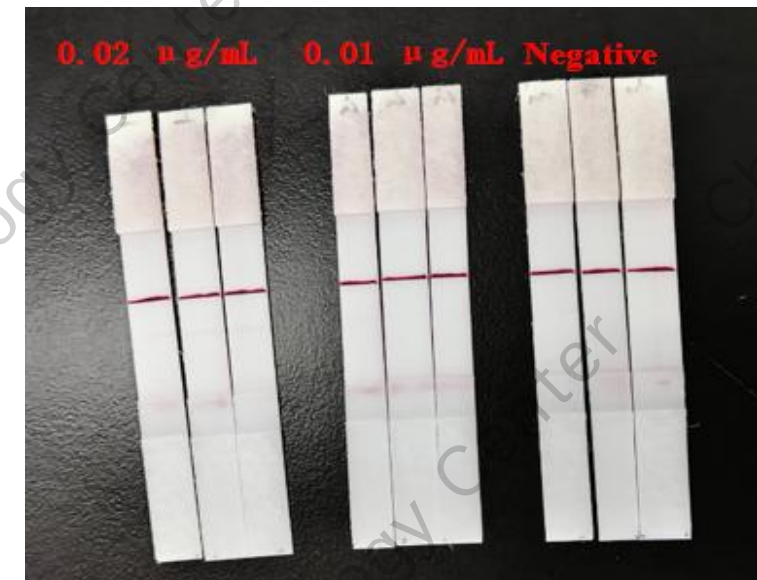


The assembled card was cut into 5 mm × 60 mm test strips using a strip cutter.



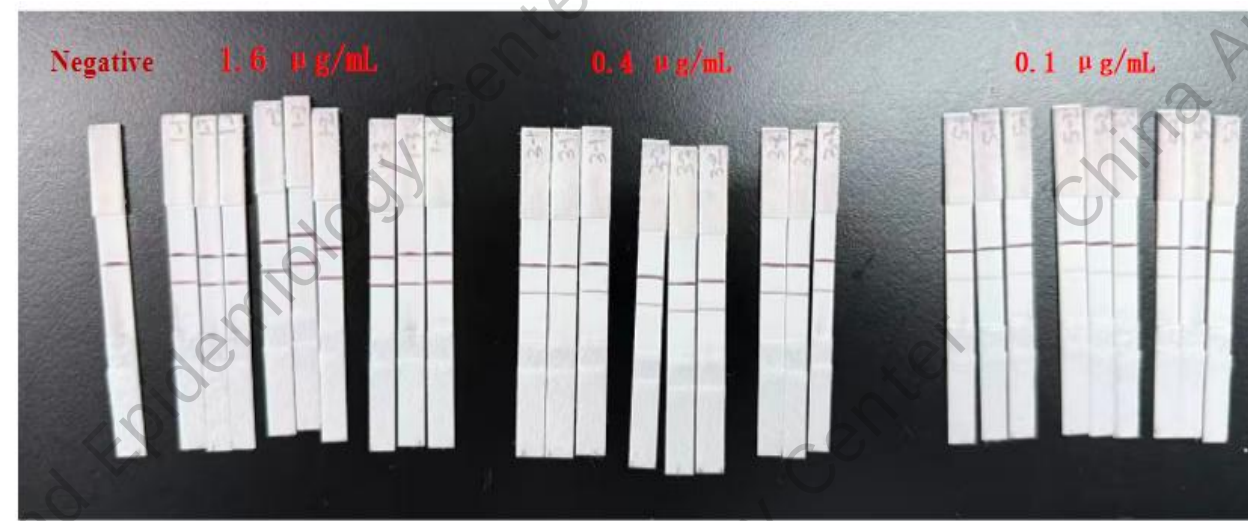
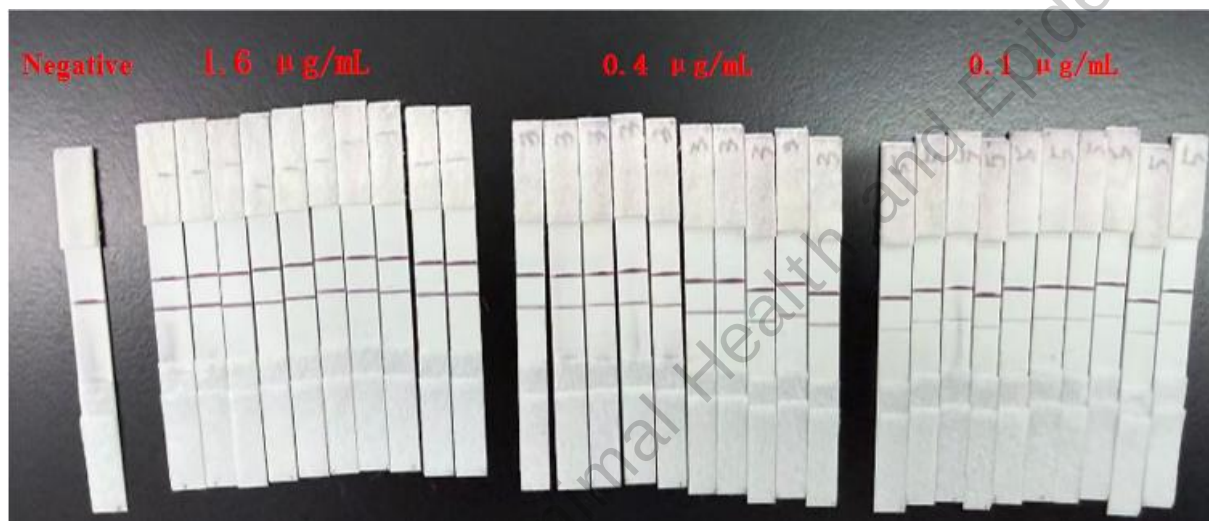


(a) Coloration results at concentrations of
0.02 µg/mL - 1.6 µg/mL



(b) Coloration results at concentrations of
0.01 µg/mL to 0.02 µg/mL

- The limit of detection (LOD) of the colloidal gold test strip was 0.02 µg/mL, and no T-line band was observed in the negative control group.



- Intra-assay test was performed 10 times and inter-assay test 9 times. Samples at high, medium and low concentrations exhibited **good repeatability** in both intra-assay and inter-assay evaluations.



(1)



(2)

This method	Thermo Fisher BOVIGAM™ TB Kit(ELISA)		Total
	Positive	Negative	
Positive	24	7	31
Negative	0	11	11
Total	24	18	42

(1) Except bovine tuberculosis-positive samples that showed colored bands on test strips, no T-line coloration was observed for other samples, indicating **good specificity** of the test strips.

(2) The coincidence rate between this method and the Vdia bovine Mycobacterium tuberculosis specific cellular immune response detection kit was 83.3%.



Thank you!

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WOAH Regional Hands-on Training on Zoonotic TB Diagnosis
Qingdao, China P.R., 14 – 18 September 2026

