世界动物卫生组织
World Organisation for Animal Health

中国兽医药品监察所
China Institute of Veterinary Drug Control
国家动物布鲁氏菌病参考实验室
National Reference Laboratory for Animal Brucellosis

2nd WOAH Regional Training Workshop on Brucellosis Diagnosis

检测实验标准操作规程

Standard Operating Procedure (SOP) for Detection Experiments

丨 Whole milk ring test (MRT) of dairy cattle

1 Equipment

Micropipette, sterile pipette suction head, sterile test tube with inner diameter of 1cm.

2 Reagents

Commercialized Brucella whole milk ring test antigen

3 Milk sample

The tested milk sample shall be fresh whole milk or mixed milk; When taking milk samples, the breast of the cow should be washed with warm water and wiped dry, and then the milk (collected in the middle section for testing) should be squeezed into clean containers; The milk samples collected shall be tested within the same day in summer.

4 Operation method

4.1 Balance the milk sample and Brucella whole milk ring test antigen to room temperature.

4.2 Take 1000 μ L of milk sample and add it to the sterile agglutination test tube.

4.3 Add 50 μ L of Brucella whole milk ring test antigen which is fully shaken and mixed evenly into the milk sample and fully mix.

4.4 Incubate in 37 ℃~38 ℃ water bath for 60 min.

4.5 After incubation, take out the test tube without shaking, and immediately judge.

5 Result judgment

The results are determined as follows:

a) Strong positive reaction (+++), the milk fat on the upper layer of the milk column forms an obvious red band, the milk column is white, and the critical value is clear;

b) Positive reaction (++), the ring of the cream layer is red, but not significant, and the milk column is slightly colored;

c) Weak positive reaction (+), the color of the annulus of the milk fat layer is light, but slightly darker than the color of the milk column;

d) Suspected reaction (s): the color of the band in the cream layer is not obvious, the boundary between the band and the milk column is not clear, and the milk column does not fade.

e) Negative reaction (I): there is no change in the upper layer of milk column, and the color of milk column is uniform.

Ⅱ Serum Agglutination Test (SAT)

1 Macro-method

1.1 Equipment

Glass test tube、test tube rack、micropipette、sterilized pipette tips and incubator（37 ℃ ±1℃）

1.2 Reagent

Commercialized serum agglutination test antigen、Brucella standard positive serum、Brucella standard negative serum、sample.

The diluent used to dilute serum and antigen when testing bovine serum samples is normal saline containing 0.5% carbolic acid.

The diluent used to dilute serum and antigen when testing sheep serum samples is 10% sodium oxide solution of 0.5% carbolic acid.

1.3 Test procedure

1.3.1 Collect and separate test serum samples according to routine methods.

1.3.2 Dilution of test serum samples：

a）Take cattle, horses, deer and camels serum samples for example, each serum is used with 4 agglutination tubes;

b）Add 960 μ L diluent after the first tube is marked;

c）Add 500 μ L diluent into each of the second to the fourth tubes respectively；

d）Add 40 μ L of serum into the first tube and mix well；

e）Take 500 μl of mixed liquid from the first tube and add it into the second tube, and then fully mix it. Diluting using the above method until the fourth tube, and discard 500 μl of mixed liquid from the fourth tube.

f）After dilution, the serum dilution from the 1st to the 4th tube is 1:25, 1:50, 1:100 and 1:200 respectively;

g）The dilution method of serum from sheep and pig is basically the same as the above. The difference is that 980 μl diluent and 20 μl test serum are added to the first tube, and the serum dilution is 1:12.5, 1:25, 1:50 and 1:100 respectively.

1.3.3 Take 500 μl of antigen reagent diluted according to the requirements of the instructions and add it to the diluted serum of the above tubes, shake and mix it well. Then, the sheep and pig serum dilution is changed to 1:25, 1:50, 1:100 and 1:200 respectively, and the dilution of cattle, horses, deer and camels serum sample is changed to 1:50, 1:100, 1:200 and 1:400 respectively.

For large-scale quarantine, only two serum dilutions (final dilutions after antigen addition) can be used, i.e. 1:50 and 1:100 for cattle, horses, deer and camels, and 1:25 and 1:50 for

pigs, goats, sheep and dogs.

1.3.4 Positive serum control, negative serum control and antigen control should be set for each test, as follows:

a）Negative serum control：After the freeze-dried negative serum is diluted to the specified volume according to the instructions, the method of dilution and antigen addition in the control test is the same as that of the tested serum;

b） Positive serum control：After the freeze-dried positive serum is diluted to the specified volume according to the instructions, the method of dilution and antigen addition in the control test is the same as that of the tested serum;

c）Antigen control：After diluting the antigen according to the instructions, take 500 μ L and add 500 μ L diluent to observe whether the antigen has self-coagulation.

2.3.5 Incubate the test tubes after sample adding in an incubator (37 ℃± 1 ℃) for 18 hours to 24 hours, take it out for inspection and record the results.

2.4 Result Interpretation and processing

2.4.1 Degree of agglutination reaction

The degree of agglutination reaction shall be judged according to the reference turbidimetric tube (see Appendix A for the preparation method), which shall be recorded as "++++", "+++", "++", "+" and "-" respectively, and determined according to the following instructions:

"++++" The thallus is completely agglutinated, 100% precipitation, and upper liquid is 100% clear;

"+++" The thallus is completely agglutinated, and upper liquid is 75% clear;

"++" The thallus agglutination is significant, and upper liquid is 50% clear;

"+" The thallus agglutination could be observed，and upper liquid is 25% clear;

"-"No agglutination could be observed，and liquid is uniform and turbid.

2.4.2 Conditions for results and Result Interpretation

When the positive serum has complete agglutination (++++), but the negative serum has no agglutination (-), and the antigen control has no self-agglutination (-), the test result is valid, and the test result is determined according to the following：

a) When "++" or above agglutination occurs in 1:100 serum dilution of cattle, horses, deer and camels, and 1:50 serum dilution of pigs, goats, sheep and dogs, the test serum sample is judged as positive;

b) When "++" or above agglutination occurs in 1:50 serum dilution of cattle, horses, deer and camels, and 1:25 serum dilution of pigs, goats, sheep and dogs, it is judged as suspicious.

2.4.3 Treatment of suspicious results

The domestic animals with suspicious results should be retested after 30 days. If the results are still suspicious, the cattle and sheep should be judged as positive. If the re-test results of pigs and horses are still suspicious, and the farm animals have no clinical symptoms and a large number of positive infected animals, the animals should be judged as negative.

Non-specific reactions occasionally occur in pig serum samples, which should be determined in combination with epidemiological investigation, CFT and differential diagnosis if necessary, to eliminate cross agglutination reaction of Yersinia spp.

Appendix A

Preparation method of reference turbidimetric tube for serum agglutination test

Turbid tubes shall be prepared for each test as the basis for determining the degree of agglutination reaction. First dilute the diluted working concentration of antigen with the same amount of diluent, and then prepare the turbidimetric tube according to Table 1.

Table 1 Preparation of reference turbidimetric tube

Tube number	2 times diluted antigen solution of working concentration/ μL	Diluent/μL	Clear brightness /%	Record mark
1	0	1000	100	++++
2	250	750	75	+++
3	500	500	50	++
4	750	250	25	+
5	1000	0	0	—

Results of Serum Agglutination Test (Macro-method)

Type of serum: Incubator temperature:

Reagent 1: Brucellosis serum agglutination test antigen Supplying organization: China Institute of Veterinary Drug Control

Reagent 2: Brucellosis standard positive serum Supplying organization: China Institute of Veterinary Drug Control

Reagent 3: Brucellosis standard negative serum Supplying organization: China Institute of Veterinary Drug Control

Diluent: Ambient temperature:

Reagent Name	Final dilution				
	1:25	1:50	1:100	1:200	1:400
Positive control serum					
Negative control serum					
Control Antigen					
Sample 1					
Sample 2					
Sample 3					

Degree of Agglutination Reaction:

"++++" The thallus is completely agglutinated, 100% precipitation, and upper liquid is 100% clear;

"+++" The thallus is completely agglutinated, and upper liquid is 75% clear;

"++" The thallus agglutination is significant, and upper liquid is 50% clear;

"+" The thallus agglutination could be observed，and upper liquid is 25% clear;

"-" No agglutination could be observed， and liquid is uniform and turbid.

Ⅲ Complement Fixation Test (CFT)

1 Preparation of 2.5% sheep red blood cell suspension

Take 5mL of 4% red blood cell solution and wash it three times with twice the volume of physiological saline. Centrifuge at 2000 rpm for 10 minutes each time. Take the washed red blood cells and add them to 8 mL of physiological saline to prepare a final concentration of 2.5% red blood cell suspension for later use.

2 Preparation of standard hemolysis wells

2.1 Preparation of 100% hemolytic solution

Take 0.5 mL of 2.5% sheep red blood cell suspension in a 5 mL centrifuge tube, centrifuge at 2000 rpm for 10 min, discard the supernatant, resuspend the cell pellet in 2 mL distilled water, shake vigorously to completely dissolve the red blood cells, and then add 0.5 mL of 4.5% Sodium Chloride Solution to obtain 100% hemolysis solution.

2.2 Preparation of 100% non-hemolytic solution

Take 0.5 mL of 2.5% sheep red blood cell suspension in a 5 mL centrifuge tube, then add 2.5 mL of physiological saline and mix well. This is 100% non hemolytic solution.

2.3 Preparation of standard hemolysis wells

Take a 96 well U-shaped bottom micro reaction plate, and then use a pipette to add 100% hemolytic solution and 100% non-hemolytic solution to each well according to the amount shown in Table 1. After adding each solution, place the reaction plate on an shaker and mix well for 30 seconds to prepare a standard hemolysis well for future use.

Table1 Preparation of standard hemolysis wells（Unit：μL）

	Well number				
	1	2	3	4	5
100% Hemolysis Solution	100	75	50	25	0
100% Non-hemolytic solution	0	25	50	75	100
Hemolysis degree (%)	100	75	50	25	0

3 Determination of hemolysin potency

3.1 Dilution of hemolysin

As hemolysin contains an equal amount of glycerol, 0.1mL of hemolysin was transferred to 4.9 mL of physiological saline using a pipette, resulting in a 1:100 dilution. Take 0.4 mL of 1:100

dilution solution and add it to 1.6 mL of physiological saline, which is a 1:500 dilution solution. Then take 0.1 mL of 1:500 dilution solution and add it to 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, 0.6 mL, 0.7 mL, 0.8 mL, 0.9 mL and 1.0 mL of physiological saline solution, respectively, to obtain dilutions of 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:3500, 1:4000, 1:4500, 1:5000 and 1:5500.

3.2 Dilution of complement

Dissolve freeze-dried complement in 1 mL of sterile water using a pipette, and then transfer it into a 15 mL centrifuge tube containing 9 mL of physiological saline, resulting in a 1:10 dilution of complement.

3.3 Determination of hemolysin potency

Take a 96 well U-shaped bottom microreactor plate and use a pipette to add 2.5% red blood cells, 1:10 diluted complement, and physiological saline to each well according to the amounts shown in Table 2. After adding each solution, mix the reaction plate in an oscillator for 30 seconds, then the plates are incubated at 37°C ± 2°C for 30 minutes（water bath）. The results are read after the plates have been centrifuged at 1000 g for 10 minutes at 5°C ±3°C . The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis and record the results in Table 2. Hemolysin control is simultaneously set up.

3.4 Calculation of hemolysin potency

The hemolysin control did not cause hemolysis, indicating the validity of the experiment. According to the results in Table 2, the maximum dilution of hemolysin that can completely lyse 25 μL of 2.5% sheep red blood cell suspension is one unit of hemolysin potency. Two units of hemolysin are required for use.

Table 2　Dilution of hemolysin（Unit：μL）

Centrifuge tube number	1	2	3	4	5	6	7	8	9	10	11
Dilution of hemolysin	1:500	1:1000	1:1500	1:2000	1:2500	1:3000	1:3500	1:4000	1:4500	1:5000	1:5500
1:500 diluted hemolysin	100	100	100	100	100	100	100	100	100	100	100
The amount of physiological saline	/	100	200	300	400	500	600	700	800	900	1000

Table 3　Determination of hemolysin potency（Unit：μL）

Well number	1	2	3	4	5	6	7	8	9	10	11	Hemolysin control
Dilution of hemolysin	1:500	1:1000	1:1500	1:2000	1:2500	1:3000	1:3500	1:4000	1:4500	1:5000	1:5500	1:100
The amount of hemolysin used	25	25	25	25	25	25	25	25	25	25	25	25
The amount of 2.5% sheep red blood cell suspension	25	25	25	25	25	25	25	25	25	25	25	25
The amount of 1:10 diluted complement	25	25	25	25	25	25	25	25	25	25	25	/
The amount of physiological saline	50	50	50	50	50	50	50	50	50	50	50	75
Incubate at 37°C ± 2°C for 30 minutes（water bath）												
Result (degree of hemolysis)												

4 Determination of complement potency

4.1 Dilution of complement

Dissolve freeze-dried complement in 1 mL of sterile water using a pipette, and then transfer it into a 15 mL centrifuge tube containing 9 mL of physiological saline , resulting in a 1:10 dilution of complement.

4.2 Dilution of two units of hemolysin

According to the results of hemolysin potency determination, ___mL of hemolysin stock solution was taken using a pipette and added to ___mL of physiological saline, resulting in a 1: dilution (two units of hemolysin).

4.3 Dilution of antigen

According to the instructions, the antigen potency is 1:150. In actual use, it should be diluted at 75% of the potency stated in the instructions, i.e. 1:112.5. Therefore, add 0.1mL of antigen to 11.15mL of physiological saline.

4.4 Determination of complement potency

Take a 96 well U-shaped bottom micro reaction plate and use a pipette to add 1:10 diluted complement, antigen, and physiological saline to each well according to the amounts shown in Table 3. After each solution is added, place the reaction plate on a shaker and mix for 30 seconds, then place it in a water bath at 37°C ± 2°C for 30 minutes. Add 25 μL of 2.5% sheep red blood cell suspension and two units of 25 μL hemolysin to each well according to the amount shown in Table 3. Place the reaction plate on the vortex shaker again and mix well for 30 seconds, then the plates are incubated at 37°C ± 2°C for 30 minutes（water bath）. The results are read after the plates have been centrifuged at 1000 g for 10 minutes at 5°C ±3°C. The degree of haemolysis is compared with the standard hemolysis well, and record the results in Table 4.

Table 4　Determination of complement potency （Unit：μL）

component	Well number											
	1	2	3	4	5	6	7	8	9	10	11	Control
1:10 diluted complement	5	6.5	8	9.5	11	12.5	14	15.5	17	18.5	20	0
One unit antigen for working	25	25	25	25	25	25	25	25	25	25	25	25
physiological saline	45	43.5	42	40.5	39	37.5	36	34.5	33	31.5	30	50
Incubate at 37°C ± 2°C for 30 minutes（water bath）												
two units of hemolysin	25	25	25	25	25	25	25	25	25	25	25	25

2.5% sheep red blood cell suspension	25	25	25	25	25	25	25	25	25	25	25	25
Incubate at 37°C ± 2°C for 30 minutes（water bath）												
Result (degree of hemolysis)												

4.5 Calculation of complement potency

The minimum complement amount that can completely lyse 25 μL of 2.5% sheep red blood cells suspension in the presence of two units of hemolysin is complement potency. According to the results in Table 3, calculate the dilution ratio of the original complement during use using the formula: (complement base solution concentration/the amount of 1:10 complement) × 25 × 90%=dilution ratio of the working complement. The dilution ratio of complement in this batch should be (10/___) × 25 × 90%=____, which is 1:____ dilution for use.

5 CFT for samples testing

5.1 Dilution of working antigen

Use 4.3 diluted antigen.

5.2 Dilution of complement

According to the 4.5 results, dilute the complement to 1 unit of complement using physiological saline, that is, 1 mL of complement is added to ____mL of physiological saline.

5.3 Dilution of hemolysin

Use two units of hemolysin diluted with 4.2.

5.4 Test procedure (example)

The undiluted test sera and appropriate working standards should be inactivated for 30 minutes in a water bath at 60°C ± 2°C. If previously diluted with an equal volume of physiological saline, these sera could be inactivated at 58°C ± 2°C for 50 minutes. Usually, only one serum dilution is tested routinely (generally 1/4 or 1/5 depending on the CF procedure chosen), but serial dilutions are recommended for trade purposes and when clinical signs have been reported in order to detect prozone.

Using standard 96-well microtitre plates with round (U) bottoms, the technique is usually performed as follows:

5.4.1 Volumes of 25 μL of diluted inactivated test serum are placed in the well of the first, second and third rows. The first row is an anti-complementary control for each serum. Volumes of 25 μL of CFT buffer are added to the wells of the first row (anti-complementary controls) to compensate for lack of antigen. Volumes of 25 μL of CFT buffer are added to all other wells except those of the second row. Serial doubling dilutions are then made by transferring 25 μL volumes of serum from the third row onwards; 25 μL of the resulting mixture in the last row are discarded.

5.4.2 Volumes of 25 µL of antigen, diluted to working strength, are added to each well except in the first row.

5.4.3 Volumes of 25 µL of complement, diluted to the number of units required, are added to each well.

5.4.4 Control wells containing:

a) diluent only,

b) complement + diluent,

c) antigen + complement + diluent,

are set up to contain 75 µL total volume in each case.

A control serum that gives a minimum positive reaction should be tested in each set of tests

to verify the sensitivity of test conditions.

5.4.5 The plates are incubated at 37°C ± 2°C for 30 minutes（water bath） , and a volume of 50 µL of sensitised SRBCs is added to each well. The plates are re-incubated at 37°C ± 2°C for 30 minutes（water bath）.

5.4.6 The results are read after the plates have been centrifuged at 1000 g for 10 minutes at 5°C ±3°C to allow unlysed cells to settle. The degree of hemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The absence of anti-complementary activity is checked for each serum in the first row.

5.4.7 Standardisation of results of the CFT

A unit system that is based on the WOAHISS exists for the standardisation of results. This serum contains 1000 ICFTU per ml. If this serum is tested in a given method and gives a titre of, for example 200 (50% haemolysis), then the factor for an unknown serum tested by that method can be found from the formula: 1000 × 1/200 × titre of test serum = number of ICFTU of antibody in the test serum per ml. The WOAHISS contains specific IgG; national standard sera should also depend on this isotype for their specific complement-fixing activity.

Difficulties in standardisation arise because different techniques selectively favour CF by different immunoglobulin isotypes. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test to use the same method in order to obtain the same level of sensitivity. To facilitate comparison between countries, results should always be expressed in ICFTUs, calculated in relation to those obtained in a parallel titration with a standard serum, which in turn may be standardised against the WOAHISS.

5.4.8 Interpretation of the results:

Sera giving a titre equivalent to 20 ICFTU/ml or more are considered to be positive. Animals that have been vaccinated with B. abortus S19 or B. melitensis Rev.1 between 3 and 6 months are usually considered to be infected if the sera give positive fixation at a titre of 30 or greater ICFTU/ml when the animals are tested at an age of 18 months or older.

Ⅳ Competitive ELISA of animal Brucellosis protocol (cELISA)

Preparation of Reagents

1 Positive Control

The National Standard (1,000 IU) must be dissolved by 1 mL Physiological Saline (1,000 IU/mL), then take 320 μL concentrate and add it to another 680 μL Physiological Saline (320 IU/mL). This solution is hereafter called "Positive Control".

2 Negative Control

The Negative Control serum must be dissolved by 1 mL Physiological Saline. This solution is called “Negative Control".

3 Conjugate (Secondary Antibody)

The Conjugate Concentrate must be diluted 1:20,000 in Secondary Antibody Dilution Buffer.

Note: Diluted conjugate solution is stable for up to 8 hours at 18–26°C.

Test Procedure

All reagents must be allowed to come to 18–26°C (room temperature) before use. Mix reagents by gentle inverting or swirling.

1 Obtain coated plate(s) and record the sample position.

2 Dilution of Samples, Negative Control (NC) and Positive Control (PC) on the microdilution plate.

- Dispense 196 μL of Sample Dilution Buffer into each well.
- Dispense 4 μL of UNDILUTED Negative Control (NC) into duplicate wells.
- Dispense 4 μL of UNDILUTED Positive Control (PC) into duplicate wells.
- Dispense 4 μL of UNDILUTED samples into remaining wells.

3 Mix the content of the microwells by gently tapping the plate or use a microplate shaker.

4 Dispense 50 μL of diluted samples into each well, and dispense 50 μL of competitive mAb into the corresponding well, immediately.

5 Cover the plate and incubate 30 minutes at 37°C.

6 Remove the solution and wash each well with approximately 300 μL of Wash Solution 3 times.

Note:

- Avoid plate drying between plate washings and prior to the addition of the next reagent.
- Tap each plate onto absorbent material after the final wash to remove any residual wash

fluid.

7 Dispense 100 µL of DILUTED Conjugate into each well.

8 Incubate 30 minutes at 37°C.

9 Repeat step 6.

10 Dispense 100 µL of TMB-Substrate into each well.

11 Incubate 15 minutes at room temperature away from direct light.

12 Dispense 50 µL of Stop Solution into each well.

13 Measure and record optical densities values of samples and controls at 450 nm.

14 Calculations:

Controls

$$\overline{NCx} = \frac{NC1\ A(450) + NC2\ A(450)}{2}$$

Validity criteria

NCx > 0.8, PI(NCx) > 70%

Samples

$$PI = \frac{NCx - Sx}{NCx}$$

15 Interpretation:

• PI ≥ 50%, Positive

• PI＜20%, Negative

Ⅴ Indirect ELISA of animal Brucellosis protocol (iELISA)

Descriptions and Principles

Microplates are coated with *Brucella* lipopolysaccharide (LPS). Samples to be tested are diluted and incubated in the wells. Upon incubation of the test sample in the coated wells, *Brucella* specific antibodies form immune-complexes with *Brucella* LPS. After washing away unbound material, an anti-ruminant antibody enzyme conjugate is added which binds to any immune-complex *Brucella* LPS-Antibody. Unbound conjugate is washed away and enzyme substrate (TMB) is added. In presence of the enzyme, the substrate is oxidized and develops a blue compound becoming yellow after blocking.

Subsequent color development is directly related to the amount of antibody to *Brucella* present in the test sample. The result is obtained by comparing the sample optical density with the Positive Control mean optical density.

Preparation of Reagents

1 Positive Control

The National Standard (1,000 IU) must be dissolved by 1 mL Physiological Saline (1,000 IU/mL), then take 320 µL concentrate and add it to another 680 µL Physiological Saline (320 IU/mL). This solution is hereafter called "Positive Control".

2 Negative Control

The Negative Control serum must be dissolved by 1 mL Physiological Saline. This solution is called “Negative Control".

3 Conjugate (Secondary Antibody)

The Conjugate Concentrate must be diluted 1:20,000 in Secondary Antibody Dilution Buffer.

Note: Diluted conjugate solution is stable for up to 8 hours at 18–26°C.

Test Procedure

All reagents must be allowed to come to 18–26°C (room temperature) before use. Mix reagents by gentle inverting or swirling.

1 Obtain coated plate(s) and record the sample position.

2 Dispense 100 µL of diluted samples into each well.

3 Cover the plate and incubate 30 minutes at 37°C.

4 Remove the solution and wash each well with approximately 300 µL of Wash Solution 3 times.

Note:

• Avoid plate drying between plate washings and prior to the addition of the next reagent.

• Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.

5 Dispense 100 µL of DILUTED Conjugate into each well.

6 Incubate 30 minutes at 37°C.

7 Repeat step 4.

8 Dispense 100 µL of TMB-Substrate into each well.

9 Incubate 15 minutes (> 15 mins is strictly prohibited to avoid false-positive results) at room temperature away from direct light (Note: The plates must be put in your drawer to keep away from bright).

10 Dispense 50 µL of Stop Solution into each well.

11 Measure and record optical densities values of samples and controls at 450 nm.

12 Calculations:

Controls

$$PC\bar{x} = \frac{PC1\ A(450) + PC2\ A(450)}{2}$$

$$NC\bar{x} = \frac{NC1\ A(450) + NC2\ A(450)}{2}$$

Validity criteria

PCx ⩾ 0.8, NCx < 0.2, NCx /PCx < 10%

Samples

$$S/P\ \% = 100\ x\ \frac{Sample\ A(450) - NC\bar{x}}{PC\bar{x} - NC\bar{x}}$$

15 Interpretation:

• S/P%⩾20%, Positive

• S/P%＜20%, Negative

Ⅵ Rose Bengal Plate Agglutination Test (RBPT)

1) Bring the serum samples and antigen to room temperature (22°C ± 4°C); only sufficient antigen for the day's tests should be removed from the refrigerator.

2) Place 25–30 µl of each serum sample on a white tile, enamel or plastic plate.

3) Shake the antigen bottle well, but gently, and place an equal volume of antigen near each serum spot.

4) Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2 cm in diameter.

5) The mixture is agitated gently for 4 minutes at room temperature (22°C ± 4°C) on a rocker or three-directional agitator (if the reaction zone is oval or round, respectively).

6) Read for agglutination immediately after the 4-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day's tests are begun to verify the sensitivity of test conditions.

Ⅶ IS711 real-time PCR assay for Brucella spp

Method description

This method detects the insertion sequence IS711 of *B. melitensis, B. abortus, B. canis, B. ovis* and *B. suis*. IS711 is characteristic for *Brucella* spp. and occurs in variable numbers (5-38 copies). This test allows the rapid and sensitive detection of DNA of *Brucella* spp. from samples purified from blood, milk, lymph tissue, bone marrow and biopsies (e.g. with the QIAamp DNA Mini Kit).

A probe-specific amplification-curve in the FAM channel indicates the amplification of *Brucella* specific DNA.

This test has been validated with the Applied Biosystems® 7500 Real-time PCR System (Thermo Fisher Scientific) and tested with a LightCycler® 480 Instrument II (Roche), but is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM channel.

The test is based on real-time PCR. A specific DNA sequence of the pathogen genome is detected and amplified. The generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows a sequence-specific detection of PCR amplificates.

Storage Conditions

The components should immediately be stored at -20°C upon arrival.

Once the lyophilized components have been resuspended, they should not be exposed to temperatures above -20°C for longer than 30 minutes at a time.

Minimize the number of freeze thaw cycles by storing in working aliquots.

Required Reagents and Equipment

- Real-time PCR Instrument
- DNA extraction kit
- RNase/DNase free qPCR plasticware: PCR tubes, strips, caps, 96-well plates, adhesive films
- Pipettors and filter tips
- Vortex and centrifuge

Primer and Probe

BruF	CGCTCGCGCGGTGGAT	
BruR	CTTGAAGCTTGCGGACAGTCACC	IS711
BruP	FAM-ACGACCAAGCTGCATGCTGTTGTCGATG-BHQ1	

Sample Material

All nucleic acid samples that are suitable for PCR amplification can be used with this kit. However, sample collection of biologic material, transport, storage and processing time are critical to achieve optimal results. Please ensure the samples are suitable in terms of purity, concentration and DNA integrity.

In addition, we recommend running at least one negative control with the samples. To prepare a negative control, replace the template DNA sample by RNase/DNase free water.

To help preventing any carry-over DNA contamination, we recommend assigning independent areas for reaction set-up, PCR amplification and any post-PCR gel analysis. It is essential that any tubes containing amplified PCR product are not opened in the PCR set-up area. We also recommend the use of RNase and DNase-free plasticware/reagents, filter tips (eventually of low-retention) for all pipetting steps and a clean area to work.

Real-time PCR Detection Protocol

1. DNA sample

For each DNA sample, prepare a reaction mix according to the table below (final volume per reaction). Include sufficient reactions for positive and negative controls.

Component	Final Concentration （μmol/L）	Volume per well or tube（μL）
Upstream primer BruF	0.3	0.6
Downstream primer BruR	0.3	0.6
BruP	0.3	0.6
2×GoTag Probe qPCR Master Mix	/	10
ddH_2O	/	6.2

2. Reaction set-up

2.1. Pipette 18 μL of each mix into individual wells according to your real-time PCR experimental plate set-up.

2.2. Pipette 2 μL of DNA template into each well, according to your experimental plate setup. The final volume in each well should be 20 μL.

2.3. For negative controls, use 2 μL of RNase/DNase free water instead of DNA template.

The final volume in each well is 20 μL.

Suggested thermal cycling conditions

The table below displays a standard protocol optimized on a number of platforms. However, these conditions may be adapted to suit different machinespecific protocols.

Cycles	Temperature	Time	Notes
1	95 ℃	2 min	Polymerase activation
40	95 ℃	10 s	Denaturation
	60 ℃	35 s	Annealing/Extension*

*Fluorogenic data should be collected during this step through both FAM channel.

Data analysis

Target Ct	Positive control Ct	Negative control Ct	Result
≤38	+	-	Positive result
>38	+	-	Suspected positive result. The test for this sample should be repeated.
-	+	-	Negative result
+/-	-	+/-	Experiment failed
+/-	+	+	Experiment failed due to test Contamination.

Positive Control: Positive control template is expected to amplify earlier than Ct 26.

BruF	CGCTCGCGCGGTGGAT	
BruR	CTTGAAGCTTGCGGACAGTCACC	IS711
BruP	FAM-ACGACCAAGCTGCATGCTGTTGTCGATG-BHQ1	