



Brucellosis
Complement Fixation Test
(EU RL cold technique)
Standard Operating Procedure



SAFETY PRECAUTIONS

The laboratory shall take all precautions in order to guarantee the necessary safety, for both the operator and the environment, against the biological and chemical hazards due to the activities conducted according to this document.

1 Scope

The present document describes a standard technique aiming at detecting antibodies specific of smooth *Brucella* species (especially *B. abortus*, *B. melitensis* and *B. suis*) by the **complement fixation test** in animal sera (ruminants, equidae, suidae, camelidae and carnivores, both wild and domestic, in particular).

2 Normative references

- Bovine brucellosis, *In*: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees), 6th Edition 2008, Chapter 2.4.3, OIE, Paris, 624-659. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.03_BOVINE_BRUCELL.pdf
- Commission Decision of 10 December 2008 (2008/984/EC) amending Annex C to Council Directive 64/432/EEC and Decision 2004/226/EC as regards diagnostic tests for bovine brucellosis (*notified under document number C(2008) 7642*). Official Journal of the European Union, L 352/38-L 352/45.
- ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories.
- OIE Quality Standard and Guidelines for Veterinary Laboratories, 2nd Edition, 2008, OIE, Paris.
- *Norme Française (French Standard) NF U 47-004 - Méthodes d'analyse en santé animale, Recherche d'anticorps contre la brucellose par la micro-méthode de fixation du complément*, avril 2009, AFNOR, France.

3 Definitions

• Complement (C)

A serum molecular complex, some components of which may fix themselves to specific antigen-antibody immune complex.

• Haemolysin (H)

Serum from a hyper-immunised animal against heterologous red blood cells (RBC, erythrocytes), with a high titre of anti-red blood cells antibodies and causing *in vitro* the lysis of specific corresponding erythrocytes whenever the complement is present.

• Sensitised RBC

A mixture of pre-determined quantities of a suspension of RBC and of a specific haemolysin.

• Series of tests

Implementation of all of the analytical phases of a technique carried out continuously or intermittently, separated by short interruptions, by the same operator(s), in the same location, with the same equipment and the same reagents.

4 Principle and reaction

4.1 Principle

The heterologous complement is set in a mixture of antigen-test serum. Once the specific antibody-antigen immune complexes are formed, the complement fixes to these complexes. This reaction is revealed by adding a second immune system: erythrocytes-haemolysin (sensitised RBC).

Indigenous complement naturally present in the test serum is destroyed via heat inactivation.

The heterologous complement that was not fixed to the first complexes, will fix to the sensitised RBC, thus causing the lysis of RBC to an extent that depends on the quantity of the complement that was not used on the first stage.



The degree of haemolysis, observed through the colouring of the reaction medium (after centrifugation or sedimentation), is inversely proportional to the titre of specific antibodies originally present in the serum.

4.2 Reaction

4.2.1 Method

The method used is the cold fixation in micro-titration plates.

4.2.2 Antigen (Ag)

The antigen is a 0.5 % phenol-saline suspension of inactivated *Brucella abortus* biovar 1, strain 99.

The supplier must certify that the activity of the antigenic preparation has been standardised against the international primary (OIEISS) or National secondary standard, with the technique established in this document and according to OIE requirements. Antigen is used at a volume of 25 µl.

4.2.3 Test sera

Sera are tested, once inactivated, at a 25-µl volume at a 1/4 dilution or in any other dilution according to the specifications requested for the test. If a prozone phenomenon, together with a high titre of antibodies in the serum is suspected (*i.e.*: abortions), it is advisable that all sera be tested at the following dilutions 1/4, 1/8, 1/16, 1/32 and 1/64 (9.1.6). On the other hand, if titres under 20 international units per millilitre (IU/ml) need to be identified, it is suggested that the serum be tested as of dilution 1/2.

4.2.4 Control sera

Control sera are used, once inactivated, at a volume of 25 µl, at a 1/4 dilution for the negative control serum and at dilutions around the expected titre for the positive control serum.

4.2.5 Complement (C)

Complement (from guinea-pig) is used at a volume of 25 µl and contains six 50% haemolytic units of complement (CH50).

4.2.6 Sensitised Sheep Red Blood Cells (SRBCs)

The sensitised-SRBCs used at a volume of 50 µl, are a mixture of equal volumes of:

- a sheep red blood cells (SRBCs) suspension at 2.5 %, and of
- a rabbit haemolysin dilution at a titre of twice the minimum concentration required to produce 100 % lysis of SRBCs in the presence of a titrated solution of guinea-pig complement (two 100%-haemolytic-units).

4.3 Controls

4.3.1 Serum Control

The absence of the anti-complementary activity of the test serum is checked in a corresponding "serum-control well". In this well, the test is performed without antigen, its volume being replaced by the same volume of the diluent. This control is performed for each test serum. In this technique, control sera are established at least for the 1/4 dilution (and for the 1/2 dilution if appropriate).

4.3.2 Antigen Control

The absence of the anti-complementary activity of the antigen used is checked in an "antigen-control well". In this well, the test is performed without serum, this latter being replaced by the same volume of diluent. This control is performed once for each series of tests.

4.3.3 Complement control

The activity of the complement on the sensitised SRBC is checked in a "complement-control well". In this well, the test is performed with the complement and the sensitised SRBCs only, the antigen and the serum being both replaced by identical volumes of diluent. This control is performed once for each series of tests.

4.3.4 Sensitised SRBCs control

The sensitised SRBCs' quality is checked in a "sensitised SRBCs-control well". In this well, the test is performed with the sensitised SRBCs only, the complement, the antigen, and the serum being all replaced by identical volumes of diluent. This control is performed once for each series of tests.



4.3.5 Haemolysis standard control

In order to facilitate test reading, the degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100 % lysis that are systematically created for each test series.

4.3.6 Control sera

Positive and negative control sera are both included in each series of tests.

5 Diluents, culture media, reagents and other products

5.1 Diluents

5.1.1 Veronal buffer calcium magnesium pH 7.2 (VB)

This may be prepared from tablets available commercially.

Example:

NaCl.....	8.500 g
Barbital	0.575 g
Diethylmalonylurea sodium	0.185 g
MgCl ₂ , 6 H ₂ O	0.168 g
CaCl ₂	0.028 g
Distilled or equivalent quality water	up to 1 000 ml

5.2 Culture media

Not applicable.

5.3 Reagents

5.3.1 Antigen

The antigen is available commercially, for veterinary use, to be diluted and stored according to the supplier's instructions. This antigen should not be exposed to temperatures ≤ 1 °C.

5.3.2 Freeze-dried guinea-pig complement

The complement is available commercially, and should be reconstituted according to the supplier's instructions.

For the CFT, the complement should be diluted according to the result of the complement titration (Annex A). If the complement is not stabilised, it should be titrated at each series of tests. If the complement is stabilised the titration should be performed for each new batch, or better each new vial. In any case, complement titration at each series of tests provides the best reliability of the final results.

The reconstituted complement, if not used immediately, must be stored at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$, until the performance of the series of tests on the same day, and for any volume left, at $\leq -16^{\circ}\text{C}$ or $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ according to the supplier's instructions.

5.3.3 Sheep erythrocytes at 50 %

Sheep erythrocytes (SRBCs) at 50 % are to be diluted to 1/20 (final concentration: 2.5 %) (9.1.1) in VB (5.1.1).

5.3.4 Rabbit haemolytic anti-SRBC serum (haemolysin)

Rabbit haemolytic anti-SRBC serum should be diluted according to the titration performed for each new batch (Annex B).

5.3.5 Control sera

5.3.5.1 Positive control serum with a known titre (commercial or lab. prepared).

5.3.5.2 Negative control serum (commercial or lab. prepared).

5.4 Other products

5.4.1 Water

The chemical and bacteriological quality of the water used to prepare the different reagents shall be verified. It must meet the requirements of the supplier and/or those imposed by the laboratory. These



requirements shall enable a satisfactory implementation of the technique described in the present document.

6 Equipment and plastic/glass ware

Conventional serology laboratory equipment and in particular:

- 6.1 Temperature-controlled incubator set at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
- 6.2 Water bath (circulating water bath if possible) set at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
- 6.3 Water bath (circulating water bath if possible) set at $59^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- 6.4 Temperature-controlled refrigerator at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$.
- 6.5 Temperature-controlled freezer at $\leq -16^{\circ}\text{C}$.
- 6.6 Centrifuge, refrigerated if possible (allowing adequate acceleration)
- 6.7 Distribution and dilution device having a suitable volume range and accuracy
- 6.8 Disposable microplates (96 well round U bottomed) with lid or cover (plastic or adhesive)
- 6.9 Test tubes and racks.
- 6.10 Plate-reading mirror (eventually).
- 6.11 Timer or chronometer.

7 Sampling

Not applicable.

8 Preparation of the sample for analysis

The preparation of the sample shall comply with the requirements of the OIE Manual.

9 Operating procedure

9.1 Test

9.1.1 Preparation of erythrocytes

SRBC (5.3.3) are diluted to 2.5 % (final dilution) in VB (5.1.1). This suspension should be prepared in a sufficient quantity to perform both the complement titration and the tests.

9.1.2 Preparation of the haemolysin

The haemolytic serum (5.3.4) is diluted according to the results of the titration (Annexe B item B.1) in VB (5.1.1). This suspension should be prepared in a sufficient quantity to perform both the complement titration and the tests.

9.1.3 Preparation of the antigen

Dilute the antigen (5.3.1) according to the supplier's instructions. This suspension should be prepared in a sufficient quantity to perform both the complement titration and the tests.

9.1.4 Complement titration (see Annex A)

9.1.5 Heat-inactivation of test and of control sera

Inactivate test and control sera (5.3.5) undiluted for 30 min in water bath at $59^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

9.1.6 Dilution of test sera and of control sera

- 25 μl of each inactivated test-serum is mixed to 75 μl of VB (5.1.1) in an intermediate plate to prepare the 1/4 dilution (or 50 μl / 50 μl for a 1/2 dilution).
- 25 μl of this 1/4 dilution (or 1/2 dilution) 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 VB (5.1.1) are added to the wells of the first row (anti-complementary controls) to compensate for lack of antigen.
- Volumes of 25 μl of VB (5.1.1) 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.
- Do the same for the positive control serum.
- The negative control serum is tested at 1/4 dilution only.

9.1.7 Distribution of the antigen, the buffer and of the complement

	Diluted serum	VB	Diluted antigen	C 6H ₅₀
Anti-complementary control well (dilution 1/4)	25 µl	25 µl	—	25 µl
Test wells (dilutions 1/4 - 1/256)	25 µl	—	25 µl	25 µl

9.1.8 Preparation of controls

	VB	Diluted antigen	C 6 H ₅₀
Antigen control	25 µl	25 µl	25 µl
Complement control	50 µl	—	25 µl
Sensitised SRBCs control	75 µl	—	—

9.1.9 Reaction

Shake the plates, cover them and place them at 5°C ± 3°C overnight (16-20 h.).

9.1.10 2nd step (haemolysis / haemolysis inhibition)

9.1.10.1 Preparation of the sensitised SRBCs: mix equal quantities of the solutions (9.1.1) and (9.1.2) that have been prepared beforehand and stored separately at 5°C ± 3°C.

9.1.10.2 Leave the mixture at room temperature for 10 min.

9.1.10.3 Take the plates out of the refrigerator and place them for 10 min. in the incubator at 37°C ± 2°C, if possible without stacking them. The sensitised SRBCs stay then 20 min. at room temperature.

9.1.10.4 Add 50 µl of the sensitised SRBCs in each well, shake the plates, cover them and place them in the incubator at 37°C ± 2°C for 30 min., if possible without stacking them.

9.2 Validation and reading

9.2.1 Centrifugation

Centrifuge plates in order to obtain the SRBCs sedimentation, e.g. 3,000 m.s⁻² and 6,000 m.s⁻² (ca. 300 g to 600 g (1 g = 9.81 m.s⁻²) for 5-10 min. Otherwise, they should be placed at 5°C ± 3°C for 2–3 hours to allow unlysed cells to settle.

9.2.2 Validation

The series of tests is validated by the result of its controls:

- Antigen control: 100 % haemolysis;
- Complement control: 100 % haemolysis;
- Sensitised SRBCs control: 0 % haemolysis;
- Negative serum control: 100 % haemolysis;
- The positive control serum is visualized at the expected titre ± one dilution.

9.2.3 Reading

The reading is made by evaluating the colour of the supernatant in comparison to a haemolysis standard control range that is prepared as follows:

Haemolysis control (%)	100	75	50	25	0
Notation	0	+	++	+++	++++
VB (µl)	0	25	50	75	100



Total haemolysis supernatant (µl)^a	100	75	50	25	0
^a prepared from 100 % haemolysis wells.					

If needed, read the results using a plate-reading mirror.

9.3 Interpretation

9.3.1 Expression of results

The raw results are expressed according to the percentage of the observed haemolysis:

	Haemolysis	Haemolysis inhibition (%)
++++:	0	100
+++:	25	75
++:	50	50
+:	75	25
0:	100	0

AC: anti-complementary activity (SRBCs not lysed in the 1/4 control well).

9.3.2 Interpretation

The end-point of the reaction is taken as the **highest dilution showing 1 + reaction or greater, i.e. 25% or more fixation.**

Interpretation is done after the conversion of titration results, expressed in international units of complement fixation per millilitre (IU/ml) established as follows:

NOTE: According to OIE and EU requirements, a result of 20 IU/ml (i.e. at least 50 % of haemolysis inhibition at 1/4 dilution) is positive.

Serum dilution	Haemolysis inhibition			
	25% (+)	50% (++)	75% (+++)	100% (++++)
1/2	8.33	10	11.67	13.33
1/4	16.67	20*	23.33	26.67
1/8	33.33	40	46.67	53.33
1/16	66.67	80	93.33	106.67
1/32	133.33	160	187	213.33
1/64	266.67	320	373.33	426.67
1/128	533.33	640	746.67	853.33
1/256	1 066.67	1 280	1 493.33	1 706.67

** European and OIE threshold of positivity (20 IU/ml).*

If preferred, the values might be rounded to the nearest integer as follows:

Serum dilution	Haemolysis inhibition			
	25% (+)	50% (++)	75% (+++)	100% (++++)
1/2	8	10	12	13
1/4	17	20*	23	27
1/8	33	40	47	53
1/16	67	80	93	107
1/32	133	160	187	213



1/64	267	320	373	427
1/128	533	640	747	853
1/256	1 067	1 280	1 493	1 707
* European and OIE threshold of positivity (20 IU/ml).				

10 Storage and disposal of samples

Each laboratory should set up the provisions for the correct storage of samples until their disposal.

Sera prepared from the whole blood samples received at the lab. must be stored at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$. It is advisable for all blood samples to be centrifuged and stored whenever possible without the clot. For long-lasting storage, it is advisable to freeze sera without clot at $\leq -16^{\circ}\text{C}$.

Decontamination and disposal of samples must be performed in accordance to in-force regulations.

11 Restitution of results

For the laboratory's clients, restitution of the results is made:

- either qualitatively (negative, positive, anti-complementary or un-interpretable) ;
- or semi-quantitatively (titre in IU/ml or anti-complementary or un-interpretable)

12 Precision

The use of positive reference material (secondary or working standard) during each series of tests enables to check the reproducibility of tests conditions. The expected titre of this reference test material must be found at more or less one dilution.

13 Analysis report

The analysis report must comply with the requirements of ISO/IEC 17025.

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Annex A

Titration of the Complement

A.1 Titration of the Complement (Six 50 % haemolytic units (6 CH₅₀))

A.1.1 Prepare a 1/100 dilution of the complement (5.3.2) in VB.

A.1.2 Distribute in tubes according to the chart below:

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	Haemolysis controls		
														H100	H0	
C dilutions (%)	0.2	0.25	0.3	0.35	0.4	0.45	0.5	0.55	0.6	0.65	0.7	0.75	0.8			
C dilutions [C 1/100 + VB] (Total vol. = 200 µl)	C (1/100) (µl)	40	50	60	70	80	90	100	110	120	130	140	150	160	400	0
	VB (µl)	160	150	140	130	120	110	100	90	80	70	60	50	40	0	0
Diluted antigen (µl)	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	
VB(µl) (in place of serum)	200	200	200	200	200	200	200	200	200	200	200	200	200	0	400	

A.1.3 Shake the tubes. Place them in the water bath at 37°C ± 2°C for 30 min.

A.1.4 Prepare the sensitised SRBCs. Mix the necessary quantities for the complement titration 20 min. before use and leave at lab. room temperature. Store the remaining volumes of haemolysin and SRBCs separately at 5°C ± 3°C until the day after.

A.1.5 Distribute 400 µl of the sensitised SRBCs **per tube**.

A.1.6 Shake the tubes. Place them in the water bath at 37°C ± 2°C for 30 min.

A.1.7 Immediately after the water bath incubation centrifuge the tubes between 3,000 m.s⁻² and 6,000 m.s⁻² (ca. 300 g to 600 g (1 g = 9.81 m.s⁻²)) for 5 to 10 min.

A.1.8 Reading of the titration

Prepare a haemolysis control at 50%: Mix at equal quantities (500 µl) the supernatant of a tube presenting a haemolysis of 0% (if not existent in the range, use VB) and the supernatant of a tube presenting a haemolysis of 100%. If there is no tube presenting a haemolysis of 100% within the range, a haemolysis control at 50% should be obtained by preparing a suspension with 400 µl of the sensitised SRBCs and 1600 µl of distilled water. Take as H50 unit the tube that presents a supernatant with the same colour as the haemolysis control at 50% thus prepared.

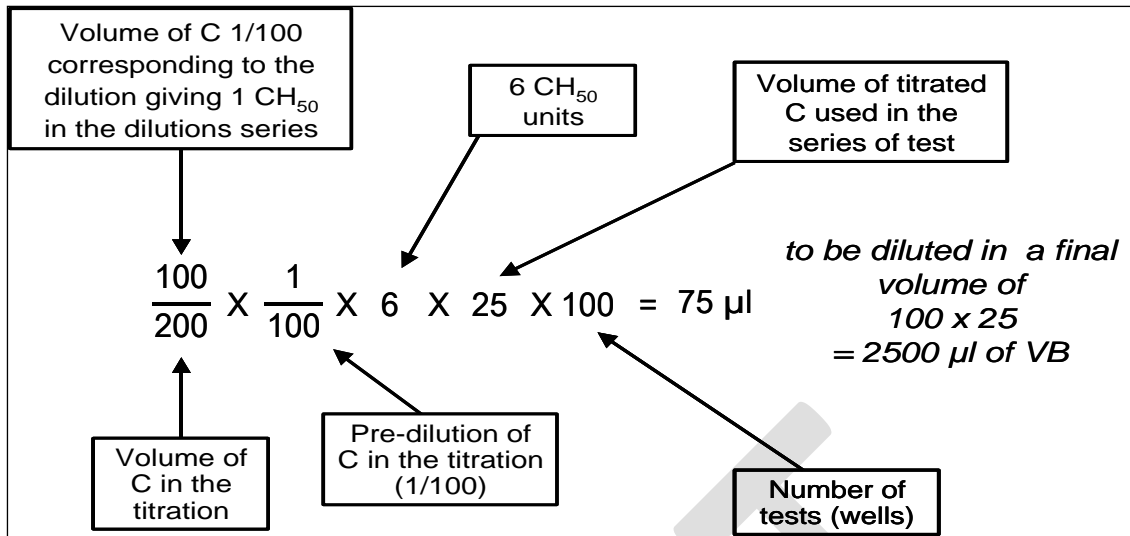
A.1.9 Interpretation: calculation of the quantity of complement required for the test.

EXAMPLE Quantity of wells = 100, each well uses 25 µl of diluted complement, that is:

25 µl × 100 = 2500 µl (total volume of diluted complement to prepare)

The haemolytic unit 50% (H50) was found for tube n° 7 (100 µl of complement at 1/100).

The test uses 6 H50, that is:



That is 75 µl of pure complement to dilute in 2425 µl of VB (5.1.1) in order to obtain a total volume of 2500 µl.

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Annex B

Haemolysin titration

B.1 Titration of haemolysin

This titration is performed in microplates, in the operating conditions of the complement fixation test. All dilutions are prepared in VB (5.1.1).

B.1.1 Prepare an initial 1/250 dilution of the Haemolysin (H). This dilution is the first of the series of dilutions presented below:

	—	1/2	1/4	1/8	1/12	1/16	1/20	1/24
Final dilution	1/250	1/500	1/1 000	1/2 000	1/3 000	1/4 000	1/5 000	1/6 000

Further dilutions may be prepared if necessary.

B.1.2 Prepare a complement dilution (5.3.2) with complement in excess (1/10 for example)

B.1.3 Prepare a SRBCs suspension at 2.5 % (9.1.1).

B.1.4 Each preparation is distributed in duplicate in a microplate following the chart below:

Wells	1 HC*	2	3	4	5	6	7	8	9
H dilutions	1/250	1/250	1/500	1/1 000	1/2 000	1/3 000	1/4 000	1/5 000	1/6 000
Diluted H (µl)	25	25	25	25	25	25	25	25	25
SRBCs 2.5 % (µl)	25	25	25	25	25	25	25	25	25
VB	75	50	50	50	50	50	50	50	50
C 1/10 (µl)	—	25	25	25	25	25	25	25	25

* HC = Haemolysin control.

Further dilutions may be prepared if necessary.

B.1.5 Shake the plate, cover it and put it in the incubator pre-set at 37°C for 30 min.

B.1.6 Centrifuge the plate at 3,000-6,000 m.s⁻² (i.e. ca. 300-600 g) for 5-10 min. (refrigerated centrifuge if possible).

B.1.7 Results

The haemolysin control (HC) shall not present any haemolysis.

The highest dilution that causes a 100 % haemolysis will determine the 100 % haemolytic unit.

In the complement fixation test, two units are used, that is the double of the quantity determined by the titration.

EXAMPLE The 1/2 000 dilution is the highest one giving a 100 % haemolysis (there is a partial haemolysis for the 1/3 000 dilution). Therefore, the dilution to use in the test is 1/1 000.





Annex C

Inhibition of the serum anti-complementary activity

This technique allows the inhibition of the anti complementary capacity of some sera.

C.1 Inhibition of the serum anti-complementary activity

- C.1.1 Prepare a solution of 5 % of serum-albumin (fraction V) in VB.
- C.1.2 Prepare the first serum dilution (1/2 or 1/4) to be tested in this solution.
- C.1.3 Incubate this dilution at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 45 min.
- C.1.4 Re-start the test at step 9.1.5.

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