



Standard Operating Procedure

Animal Brucellosis

Control of Rose Bengal, Complement Fixation and Milk Ring-test antigens



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 controlling the fulfilment of OIE and EU requirements regarding the standardisation of diagnostic antigens for the detection of antibodies specific of smooth *Brucella* species (especially *B. abortus*, *B. melitensis* and *B. suis*):

- in animal individual sera by the Rose Bengal Test (RBT) and the Complement Fixation Test (CFT);
- in bovine pooled milk samples by the Milk Ring-Test (MRT).

2 Normative references

- Brucellosis (*Brucella abortus*, *B. melitensis* and *B. suis*) (infection with *B. abortus*, *B. melitensis* and *B. suis*), *In*: OIE Terrestrial Manual, 2018, Chapter 3.1.4., Paris.
https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf
- Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products. Official Journal of the European Union 17.03.2017, L95/1-142. Text with EEA relevance. ELI:
<http://data.europa.eu/eli/reg/2017/625/oj>
- ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories.
- French Standard NF U47-310 - Animal health analysis methods - Biological reagents control for the immunological techniques used in animal health, September 2013, AFNOR, France.
- Ovine epididymitis, *In*: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2017, Chapter 2.1.4., OIE, Paris, *online version adopted by the World Assembly of Delegates of the OIE in May 2015*.



3 Definitions

- **Brucellosis**

Infection of animals due to bacteria of the genus *Brucella*, naturally in the smooth (S) phase (*B. abortus*, *B. melitensis* or *B. suis*), pathogenic for most mammal species, in which it usually induces the production of S-*Brucella*-specific antibodies.

- **National Reference Laboratory for Brucellosis (NRL)**

Laboratory officially designated as such by the authority of the corresponding Member State. According to the abovementioned Commission Decision, this NRL is responsible for:

- (1) *the approval of the results of the validation studies demonstrating the reliability of the test method used in the Member State;*
- (2) *determination of the maximum number of samples to be pooled in ELISA kits used;*
- (3) *calibration of working standards /.../;*
- (4) *quality checks of all antigens and ELISA kits batches used in the Member State;*
- (5) *following recommendations of, and cooperating with the EU reference laboratory for brucellosis.'*

- **Official Control Laboratory (OCL)**

Laboratory, usually the NRL, in charge of the official control of all antigens and ELISA kits batches used in the Member State. This activity could be assigned to another laboratory, the NRL of another Member State, in particular.

- **International standard serum**

The International standard serum for brucellosis RBT, CFT and MRT antigens is the OIE International Standard Serum (OIEISS, previously the WHO Second International anti-*Brucella abortus* Serum [ISaBS])¹.

The OIEISS contains 1 000 international complement fixation units per ml (ICFTU/ml or IU/ml)

- The RBT antigen, ready-to-use, is prepared without reference to the cell concentration, but its sensitivity must be standardised in relation to the OIEISS in such a way that the antigen produces a positive reaction with a serum dilution of 1/45 and a negative reaction with a dilution of 1/55.
- The CFT antigen, ready-to-use, must be standardised in relation to the OIEISS in such a way that the antigen produces:
 - o in the EU CFT SOP, a reaction of 50% haemolysis inhibition with a serum dilution of 1/200;

¹ Obtainable from the OIE Reference Laboratory for Brucellosis at AHVLA Weybridge, Addlestone, Surrey KT15 3NB, UK. Due to the limited stock left, the OIEISS should be restricted to the control of conventional antigens (RBT, CFT, SAT, MRT in particular) and must not be used for the control of ELISA kits.

² Obtainable from the OIE/EU Reference Laboratory for Brucellosis at ANSES Maisons-Alfort, France.

³ Obtainable from the OIE/EU Reference Laboratory for Brucellosis at ANSES Maisons-Alfort, France; or from the OIE Reference Laboratory for Brucellosis at AHVLA Weybridge, Addlestone, Surrey KT15 3NB, UK.

⁴ Tests for sterility and freedom from contamination of biological materials intended for veterinary use, In: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2017, Chapter 1.1.9., OIE, Paris.



- in any other CFT SOP, a reaction of 50% haemolysis inhibition with a serum dilution of $1/((1000 / 20) \times T)$ where T corresponds to the titre of a test serum containing 20 ICFTU/ml;

Example: with a CFT SOP for which 20 ICFTU/ml corresponds to 50% haemolysis inhibition at the 1/5 dilution of a test serum, the dilution of the OIEISS for which the antigen must produce a 50% haemolysis inhibition is: $1/((1000 / 20) \times T) = 1/((50) \times 5) = 1/250$.

- The MRT antigen must be standardised in relation to the OIEISS in such a way that the antigen produces a positive reaction with a 1/500 dilution of the OIEISS in negative milk, while a 1/1 000 dilution must be negative.
- **Secondary or National standard serum (sera)²**
Secondary or national standard serum (sera) established against the abovementioned OIEISS and containing a defined concentration of anti-*Brucella* antibody corresponding to a defined activity in brucellosis RBT, CFT or MRT.

4 Specific requirements before submission of an antigen to control

The following requirements must be fulfilled by the supplier or manufacturer before submitting any antigen batch to the OCL for control.

All RBT, CFT and MRT antigens consist in a pure and inactivated suspension of *Brucella abortus* biovar 1 Weybridge strain No 99³ or USDA strain 1119-3 in a solution containing 0.5% phenol (V/V).

Culture media used for the maintenance of the strain as well as for the production of the antigens must not favour the bacterial dissociation. In adequate conditions, the dissociation rate (R/(S+R)) must be less than 1%.

The purity control of the antigens is performed by the examination of a Gram-stained smear of the antigen. It must reveal a pure and homogenous population of Gram negative coccobacilli.

The sterility control of the antigens is performed according to the OIE requirements⁴. This control includes a medium that favours the growth of *Brucella*.

The supplier/manufacturer must be able to produce, on request, internal control certificates attesting that the abovementioned controls have been performed and that expected requirements were fulfilled.

RBT:

The antigen represents a bacterial suspension in buffered *Brucella* antigen diluent at a pH of 3.65 ± 0.05 , stained by the use of rose Bengal dye. The antigen shall be delivered ready for use and must be stored at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and not frozen.



CFT:

The antigen represents a bacterial suspension in phenol-saline [NaCl 0.85% (m/v) and phenol at 0.5% (v/v)] or in a veronal buffer. Antigens may be delivered in the concentrated state provided the dilution factor to be used is indicated on the bottle label. The antigen must be stored at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and not frozen.

MRT:

The antigen represents a bacterial suspension in phenol-saline [NaCl 0.85% (m/v) and phenol at 0.5 % (v/v)] stained with haematoxylin. The antigen must be stored at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and not frozen.

5 Sampling

The supplier or manufacturer sends the reagents in their final form (reagent, final packaging, and instructions for use) to the OCL in sufficient numbers for the performance of all tests needed by the present procedure (control of a new batch, control of a batch during the validity period).

6 Storage and disposal of batch samples

According to the instructions of the supplier/manufacturer during the validity period.

7 Standard sera and Reference materials

These materials are stored at a temperature $< -16^{\circ}\text{C}$ or freeze-dried and stored at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$.

7.1 International standard serum

The International standard serum for brucellosis RBT, CFT and MRT antigens is the OIE International Standard Serum (OIEISS).

7.2 Secondary or national standard serum (or sera)

7.3 Reference panel of positive sera

Serum samples issued from animals naturally or experimentally infected by smooth *Brucella* (*B. abortus*, *B. melitensis* or *B. suis*). These sera must be chosen in such a way that the reaction obtained is slightly above the cut-off. Bovine samples are needed for the control of MRT antigens.

7.4 Reference panel of negative sera

Serum samples issued from brucellosis free animals.



7.5 Dilution Negative serum (DNS)

A pool of at least 3 negative serum samples is recommended.

7.6 Dilution Negative milk (DNM)

A pool of at least 3 negative milk samples is recommended.

Note: materials 7.2-7.6 must be made available to any kit supplier/manufacturer on request.

7.7 Saline or phenol-saline

NaCl 0.85% (m/v) or NaCl 0.85% (m/v) and phenol 0.5% (v/v)

7.8 Magnesium Calcium Buffer (MgCa Buffer)

As foreseen in the EU CFT SOP.

8 Principle

8.1 Initial Control

This control is performed on the first batch of a new antigen, submitted to the OCL for approval, once the validation dossier has been submitted by the supplier/manufacturer to the NRL and has been approved by the latter as fulfilling the OIE requirements. Any RBT, CFT or MRT antigen is considered as validated provided that it fulfils the requirements mentioned in 4) and if adequate results are obtained in the appropriate control tests detailed hereafter.

The OCL checks the following parameters:

8.1.1 Antigen titre and Limit of detection (LOD)

See Annex A:

8.1.2 Sensitivity

- Five positive sera from the Reference panel of positive sera (7.3) tested once in the appropriate test.

For RBT and CFT, the reference positive sera are diluted in the DNS (7.5) in such a way that they contain an antibody level comparable or slightly higher than the expected limit of detection.

For MRT, the reference positive sera are diluted in the DNS (7.5) then 1/10 in DNM (7.6) in such a way that they contain an antibody level comparable or slightly higher than the expected limit of detection.

For CFT, the antigen is used at the concentration found as appropriate in test 8.1.1.

For RBT and MRT, this test is not performed if the LOD found in test (8.1.1) is not conforming to the specific requirements.

8.1.3 Specificity

- Five negative sera from the Reference panel of negative sera (7.4) tested once in the appropriate test.



For CFT, the antigen is used at the concentration found as appropriate in test (8.1.1).

For MRT, the reference negative sera are diluted 1/10 in DNM (7.6).

For RBT and MRT, this test is not performed if the LOD found in test (8.1.1) is not conforming to the specific requirements.

8.1.4 Repeatability

NA

8.2 Control of a new batch

This control is performed by the OCL on samples of any new batch to be approved in the corresponding Member State and is identical to the initial control described above (8.1)

8.3 Control of a batch during the validity period

This control is performed by the OCL in the middle of the validity period of the product. The OCL may decide not to perform this control, if the supplier/manufacturer provides the results of a corresponding internal control.

Otherwise, the OCL can decide to perform this control, if needed, especially according to information coming from the routine diagnostic laboratories.

The following parameters are checked:

- Antigen titre or LOD
- Sensitivity
- Specificity

9 Equipment and plastic/glass ware

Conventional serology laboratory equipment.

10 Operating procedure

The OCL must follow the appropriate SOP in force in the Member State.

Nevertheless, SOP must be adapted according to Annex A, where applicable.

11 Interpretation of results

The criteria for the interpretation of results are identical for the initial control, the control of a new batch and the control during the validity period.



11.1 Antigen titre and LOD

- The RBT antigen, ready to use, must produce a positive reaction with a serum dilution of 1/45 of the OIEISS (or equivalent dilution of the appropriate secondary or national standard serum) and a negative reaction with a dilution of 1/55 of the OIEISS (or equivalent dilution of the appropriate secondary or national standard serum).
- The CFT antigen, ready to use, must produce a 50% haemolysis inhibition with the 1/200 (0.5%) dilution of the OIEISS (or equivalent dilution of the appropriate secondary or national standard serum) in the EU SOP or with the adequate dilution of the OIEISS (or of the appropriate secondary or national standard serum) in any other CFT SOP (as foreseen in (3)).
- The MRT antigen, ready to use, must produce a positive reaction with a serum dilution of 1/500 of the OIEISS (or equivalent dilution of the appropriate secondary or national standard serum) and a negative reaction with a dilution of 1/1000 of the OIEISS (or equivalent dilution of the appropriate secondary or national standard serum).

11.2 Sensitivity

All five positive sera from the Reference panel of positive sera (7.3) must give a positive reaction.

11.3 Specificity

All five negative sera from the Reference panel of negative sera (7.4) must give a negative reaction.

11.4 Repeatability

NA

12 Restitution of results

The OCL provides to the supplier/manufacturer all the detailed results corresponding to the tests described in chapter 8.

13 Analysis report

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

The report clearly mentions whether the antigen complies or not with the acceptability criteria.





Annex A

Control of the antigen titre or LOD of RBT, CFT and MRT antigens

Note: The following procedures were established for the use of the OIEISS (or a secondary or national standard serum with the same titre in the respective test). They must be adapted, where necessary, if other secondary or national standard sera are used.

Control of the RBT antigen

- Prepare a 1/25 pre-dilution of the OIEISS in saline or phenol-saline (7.7);
- From this dilution, prepare dilutions 1/40, 1/45, 1/50, 1/55 and 1/100 in saline or phenol-saline (7.7) as follows:

1/25 pre-dilution of the OIEISS (mL)	1	0.5	0.5	0.5	0.5	0.25
Saline or phenol-saline (mL)	0	0.3	0.4	0.5	0.6	0.75
Final dilutions	1/25	1/40	1/45	1/50	1/55	1/100

- Perform the RBT as prescribed by the national SOP;
- A RBT antigen with the appropriate titre is used as control.

Control of the MRT antigen

- Prepare a 1/5 pre-dilution of the OIEISS in saline (7.7);
- From this dilution, prepare dilutions 1/10, 1/20, 1/50 and 1/100 in saline (7.7) as follows:

Dilution	1/5	1/10	1/20	1/50	1/100
OIEISS (mL)	0.2	-	-	-	-
Saline (mL)	0.8	0.2	0.3	0.36	0.38
1/5 pre-dilution of the OIEISS (mL)	-	0.2	0.1	0.04	0.02
Final dilution*	1/50	1/100	1/200	1/500	1/1000

* once diluted 1/10 in DNM (7.6)

- Dispatch these dilutions in 5 tubes and dilute 1/10 in DNM (7.6) for a total volume in accordance with the national SOP;
- Perform the MRT as prescribed by the national SOP;
- A MRT antigen with the appropriate titre is used as control.



Control of the CFT antigen (EU SOP)

- Prepare a 1/50 pre-dilution of the OIEISS in MgCa Buffer(7.8);
- From this dilution, prepare dilutions 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3% and 0.2% in MgCa Buffer (7.8), for instance, as follows:

OIEISS final dilution	Pre-dilution 1/50	0.8%	0.7%	0.6%	0.5%	0.4%	0.3%	0.2%
OIEISS (mL)	0.1	-	-	-	-	-	-	-
MgCa Buffer (mL)	4.9	0.6	0.65	0.7	0.75	0.8	0.85	0.9
OIEISS 1/50 (mL)	-	0.4	0.35	0.3	0.25	0.20	0.15	0.1

- Then, prepare 6 serial dilutions of the CF antigen, around the dilution prescribed by the supplier/manufacturer, in such a way that the highest concentration is at least twice the one prescribed.

Example (prescribed dilution: 1%):

Final dilutions	1/40	1/60	1/80	1/100	1/120	1/140
	2.5%	1.66%	1.25%	1%	0.83%	0.71%
Antigen (mL)	0.10	-	-	-	-	-
MgCa Buffer(mL)	3.90	0.25	0.50	0.75	1.00	1.25
Antigen 2.5% (mL)	-	0.50	0.50	0.50	0.50	0.50

- Perform the CFT as prescribed by the EU CFT SOP by testing each serum dilution with each antigen dilution;
- The titre of the antigen is given by the antigen dilution that gives 50% haemolysis inhibition (> 50% -< 50%) with the OIESS diluted 0.5%. The reading is performed by comparison to Haemolysis standard controls prepared as foreseen in the EU CFT SOP (4.3.5).
- If two titres give the expected result, the one to be chosen is the most concentrated.
- A CFT antigen with the appropriate titre is used as control.



Example:

OIEISS final dilution	0.8%	0.7%	0.6%	0.5%	0.4%	0.3%	0.2%
Control antigen	75%	< 75 %	> 50 %	50 %	< 25 %	0	0
Test antigen							
1/40	50%	25 %	0	0	0	0	0
1/60	< 75%	< 50%	25%	0	0	0	0
1/80	75%	< 75%	< 50%	25%	0	0	0
1/100	75%	75%	> 50%	50%*	< 25%	0	0
1/120	75%	75%	> 50%	50%	25%	0	0
1/140	100%	75%	> 50%	< 50%	25%	0	0

* A result included in the range [$> 50\%$ - $< 50\%$] is acceptable.