Guidelines for Brucellosis Testing in *Bison* ssp, *Bos* ssp and *Bubalus* ssp According to AHL Requirements

1 Introduction

This document is applicable to *Bison* ssp, *Bos* ssp and *Bubalus* ssp as listed in the Commission Implementing Regulation (EU) 2018/1882. Specific guidelines for *Bubalus* ssp. are in preparation.

According to the Commission Delegated Regulation (EU) 2020/689 Article 6, specific legislation and guidance for brucellosis testing will be made available:

(a) in the websites of the EURL and COM: see **Methods | EURL (anses.fr)** ;

(b) if no recommendation is provided by EURL websites, OIE Manuals have to be applied

c) if no recommendation is provided by EURL / OIE websites, Article 34 of Regulation (EU) 2017/625 should be applied:

• National rules. In absence of national rules, relevant methods recommended by NRL validated in accordance with international standards or

• Relevant methods validated with inter or intra laboratory methods validation studies

• In case of urgency, NRL (in absence, other designated lab) may use methods which have not been validated

Diagnostic methods for granting and maintaining disease freedom are laid down in Annexes III and VI (Commission Delegated Regulation (EU) 2020/689).

SECTION 1
INFECTION WITH *BRUCELLA ABORTUS, B. MELITENSIS* AND *B. SUIS*

1. Serological tests
   - (a) tests for blood samples
     - (i) buffered *Brucella* antigen tests;
     - (ii) complement fixation test (CFT)
     - (iii) indirect enzyme-linked immunosorbent assay (I-ELISA)
     - (iv) fluorescence polarisation assay (FPA)
     - (v) competitive enzyme-linked immunosorbent assay (C-ELISA)
   - (b) tests for milk samples
     - (i) ring test (MRT)
     - (ii) I-ELISA
2. Brucellin skin test (BST)

   For the testing as referred to in section 1 and 2 of Chapter 1 of Part I of Annex IV, Brucellin skin test (BST) shall only be used in ovine and caprine animals.

2 Validation of reagents and kits

All reagents and kits should be properly validated according to the EURL specifications (initial validation (see 1.1) and batch control (see 1.2) at EU or national level). **Antigens and ELISA home-made kits have to be validated.**

When the EURL has performed an initial validation (see 1.1) or a batch control (see 1.2), the possibility is given to other NRLs to use the previously validated reagents or kits.

A list of validated batches is available on the EURL website: Reagents | EURL (anses.fr)

Individual batch certificates are available on the EURL website: Approved antigens and Kit batches | EURL (anses.fr)

2.1 EURL Specifications for initial validation

The standardisation and validation are critical steps when a method is intended for routine diagnostic use in multiple laboratories. Therefore, customers can have confidence in the results produced by the test. All diagnostic assays should be validated for the species which they will be used for, according to the new OIE and EU principles and methods.

To validate a method, the manufacturer must submit a file to the National Reference Laboratory for Brucellosis (NRL) including a descriptive administrative part of the reagent(s) and a technical part. The evaluation criteria are described in different documents (see EURL website, links in table 1). The dossier will be reviewed by the NRL and a report will be returned to the manufacturer.

Table 1: Links to EURL specifications for initial validation of methods

Method	**Link to EURL SOPs for initial validation**
Buffered *Brucella* antigen tests	Brucellosis Specifications for validation of antigen for Rose Bengal Test | EURL (anses.fr)
Complement fixation test (CFT)	Brucellosis EURL Specifications for the initial validation of antigen for complement fixation test (CFT) | EURL (anses.fr)
i- and c-ELISA kits (serum)	Brucellosis Specifications for validation of ELISA kits (i-ELISA and c-ELISA) | EURL (anses.fr)
Milk Ring test (MRT)	*In progress*
FPA	No EURL recommendation (not used in EU). OIE requirements have to be applied

2.2 Batch Control (applied to kits and antigens with a previous initial validation)

For each method, protocols available in Table 2 describe a standard technique aiming at controlling the fulfilment of OIE and EU requirements regarding the control of batch and standardisation of validated reagents and ELISA kits.

Table 2: Links to EURL protocols for batch control of methods

Method	**Link to EURL SOPs for batch control**
Buffered *Brucella* antigen tests	Animal Brucellosis Control of Rose Bengal, Complement Fixation and Milk Ring-test antigens | EURL (anses.fr)
Complement fixation test (CFT)	Animal Brucellosis Control of Rose Bengal, Complement Fixation and Milk Ring-test antigens | EURL (anses.fr)
ELISA kits (serum)	Bovine Brucellosis Control of indirect ELISA kits (Individual & pooled serum samples) | EURL (anses.fr)
ELISA kits (milk)	Control of iELISA kits for the detection of antibodies specific to smooth Brucella species (especially B. abortus, B. melitensis and B. suis) in bovine tank milk. | EURL (anses.fr)
Ring test (MRT)	Animal Brucellosis Control of Rose Bengal, Complement Fixation and Milk Ring-test antigens | EURL (anses.fr)
FPA	No EURL recommendation (not used in EU). OIE requirements have to be applied

3 Standard operating protocols (SOPs) for *Bison* ssp., *Bos* ssp. and *Bubalus* ssp.

EURL protocols of methods are available on the website (see links in Table 3).

Table 3: Links to EURL standard operating protocols (SOPs)

Method	**Link to EURL SOPs for *Bison* ssp., *Bos* ssp. and *Bubalus* ssp.**
Buffered *Brucella* antigen tests	Rose Bengal Test | EURL (anses.fr)
Complement fixation test (CFT)	Complement Fixation Test | EURL (anses.fr)
i-ELISA kits	According to manufacturer instructions Indirect Enzyme Linked Immunosorbent Assay | EURL (anses.fr)
c-ELISA kits	According to manufacturer instructions Competitive Enzyme-linked immunosorbent assay | EURL (anses.fr)
Milk Ring test (MRT)	Milk Ring Test (MRT) | EURL (anses.fr)
FPA	No EURL recommendation (not used in EU). OIE requirements have to be applied

4 EURL testing guidelines applied to brucellosis control for *Bison* ssp., *Bos* ssp. and *Bubalus* ssp.

4.1 Granting the status

The Commission Delegated Regulation (EU) 2020/689 (Annex IV, part I) describes the conditions required to grant the status free from infection with *Brucella abortus, B. melitensis* and *B. suis* without vaccination may be granted with (Annex IV, part I, Chapter I, section 1) or without vaccination (Annex IV, part I, Chapter II, section 1) to an establishment keeping bovine, ovine or caprine animals.

Granting disease free status has to be done at the **level of establishments** with the tests listed in the in Annexes III (Regulation (EU) 2020/689). **It is recommended to use BBAT, i-ELISA or bulk milk tests in order to grant the status (table 4).**

Table 4: Recommended tests for granting the brucellosis status in *Bison* ssp., *Bos* ssp. and *Bubalus* ssp. (adapted from OIE)

Method	Population freedom from infection	Individual animal freedom from infection
BBAT	**+++**	++
CFT	++	++
i-ELISA	**+++**	++
c-ELISA	++	+
FPA	(not used in EU)	(not used in EU)
Bulk milk tests (i-ELISA/MRT)	**+++**	+ (tested in a pool of minimum 10 animals)

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.

4.2 Maintaining the status – Surveillance

The Commission Delegated Regulation (EU) 2020/689 (Annex IV, part I) describes the conditions required to maintain the status free from infection with *Brucella abortus, B. melitensis* and *B. suis* with (Annex IV, part I, Chapter I, section 2) or without vaccination (Annex IV, part I, Chapter II, section 2) to an establishment keeping bovine, ovine or caprine animals.

General passive surveillance is based on notifications and investigations. Specific surveillance activities include eradication programmes, disease control, pre-movement surveillance. Maintaining the disease free status has to be done at the **level of establishments** with the tests listed in the in Annexes III (Commission Delegated Regulation (EU) 2020/689). **It is recommended to use BBAT, CFT, i-ELISA or bulk milk tests in order to maintain the status (table 5).**

Table 5: Recommended tests for maintaining the brucellosis status in *Bison* ssp., *Bos* ssp. and *Bubalus* ssp. (adapted from OIE)

Method	Contribution to eradication policies	Herd/flock prevalence of infection - surveillance
BBAT	+++	+++
CFT	+++	+++
i-ELISA	+++	+++
c-ELISA	+	++
FPA	(not used in EU)	(not used in EU)
Bulk milk tests (i-ELISA/MRT)	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.

An example of surveillance flowchart is presented in Figure 1. BBAT and i-ELISA methods should be used as first line testing (most sensitive methods). In case of positive results, CFT methods should be performed in order to confirm seropositivity (specific method). If suspicion of brucellosis is confirmed, the competent authority should manage the suspicion in line with Article 9, Regulation 2020/689.

According to the level of prevalence, the choice of methods in parallel or in series should be adapted: testing in parallel will increase the sensitivity (in case of high prevalence) whereas testing in series will increase the specificity (in case of low prevalence or free zone).

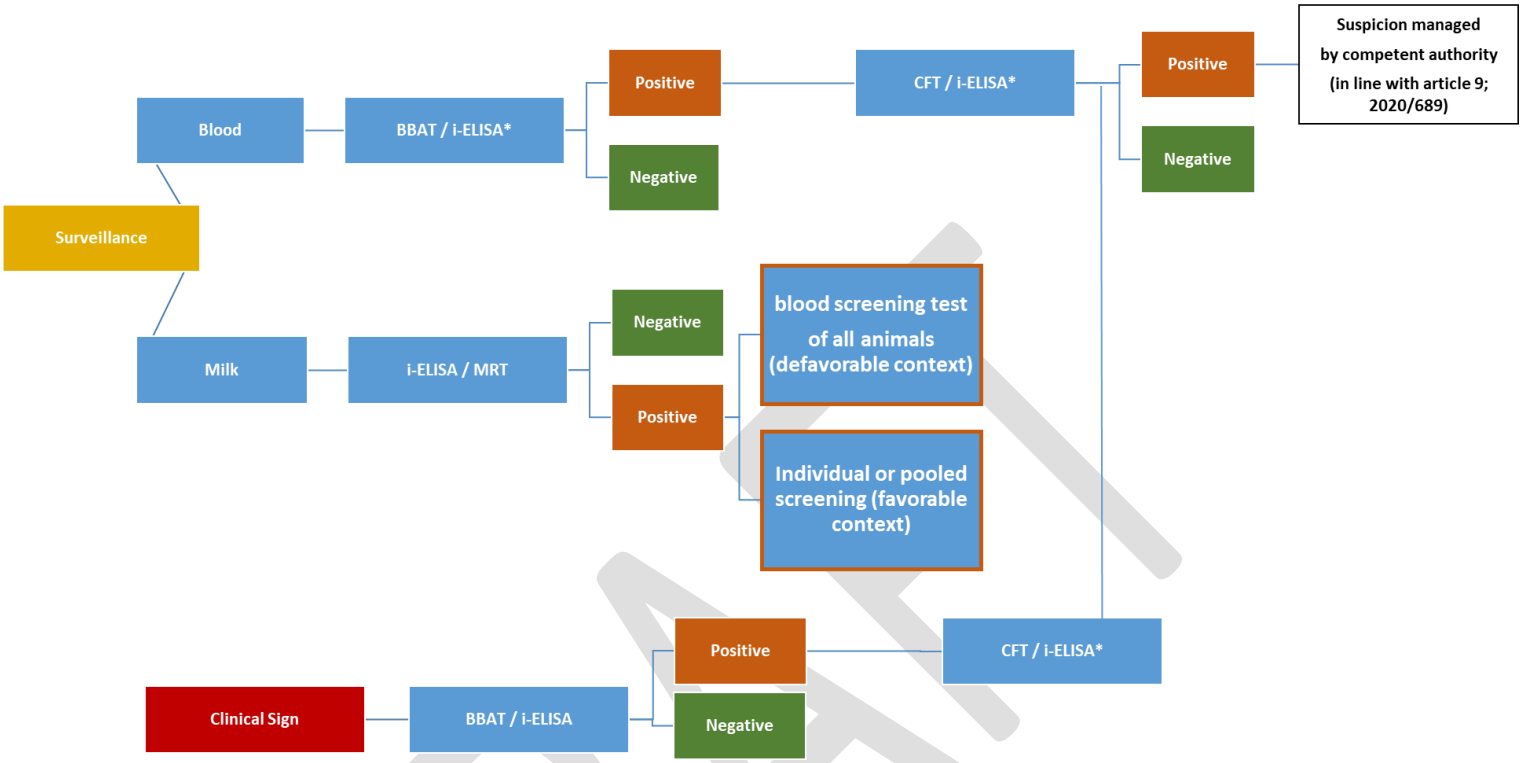

Figure 1: Example of surveillance flowchart for *Bison* ssp., *Bos* ssp. and *Bubalus* ssp. establishments in line with Regulation (EU) 2020/689

4.3 Suspension and restoring of the status

The Commission Delegated Regulation (EU) 2020/689 (Annex IV, part I) describes the situations leading to suspension of the status free from infection with *Brucella abortus, B. melitensis* and *B. suis* with (Annex IV, part I, Chapter I, section 3) or without vaccination (Annex IV, part I, Chapter II, section 3) to an establishment keeping bovine, ovine or caprine animals.

All cases of abortion as well as orchitis in cattle, sheep and goats, and pigs, should be considered as suspected brucellosis and should be investigated through the herd/flock history and submission of specimens for laboratory testing. **If suspicion of brucellosis is confirmed, the competent authority should manage the suspicion in line with Article 9, Regulation 2020/689.**

Article 9

Case definitions

1. The competent authority shall classify an animal or a group of animals as a suspected case of a listed disease or of an emerging disease when:

(a) clinical, post-mortem or laboratory examinations conclude that clinical sign(s), post-mortem lesion(s) or histological findings are indicative of that disease;

(b) result(s) from a diagnostic method are indicating the likely presence of the disease in a sample from an animal or from a group of animals; or

(c) an epidemiological link with a confirmed case has been established.

2. The competent authority shall classify an animal or a group of animals, as a confirmed case of a listed disease or of an emerging disease when:

(a) the disease agent, excluding vaccine strains, has been isolated in a sample from an animal or from a group of animals;

(b) an antigen or nucleic acid specific to the disease agent that is not a consequence of vaccination has been identified in a sample from an animal or from a group of animals showing clinical signs consistent with the disease or an epidemiological link with a suspected or confirmed case; or

(c) a positive result from an indirect diagnostic method that is not a consequence of vaccination has been obtained in a sample from an animal or from a group of animals showing clinical signs consistent with the disease or an epidemiological link with a suspected or confirmed case.

3. Disease specific definitions of a suspected case and a confirmed case of listed diseases are laid down for terrestrial animals in Annex I and for aquatic animals in point 3 of Section 5 of Chapters 1 to 6 of Part II of Annex VI.

4. In the absence of disease specific definitions as provided for in paragraph 3, the criteria laid down in paragraphs 1 and 2 shall apply to definitions of a suspected case and a confirmed case of listed diseases and, if relevant, emerging diseases.

According to the Commission Delegated Regulation (EU) 2020/689 (Annex IV, part I, Chapter I, section 3), the status free from infection with *Brucella abortus, B. melitensis* and *B. suis* without vaccination of an establishment keeping bovine, ovine or caprine animals must be suspended if:

(a) one or more of the requirements set out in Section 2 (see 3.2) are not fulfilled; or

(b) a case of infection with *Brucella abortus, B. melitensis* and *B. suis* is suspected in a bovine, ovine or caprine animal kept in the establishment.

The clinical signs are not pathognomonic and unequivocal diagnosis of *Brucella* infections can be made only by the isolation or identification of *Brucella*. In situations where bacteriological examination is not practicable, diagnosis should be based on molecular or immunological methods, including brucellin.

Additional optional protocols are available on the EURL website (Table 6), in order to investigate suspected cases. These protocols are not mandatory.

Table 6: Links to optional EURL standard operating protocols (SOPs) in case of suspected brucellosis in *Bison* ssp., *Bos* ssp. and *Bubalus* ssp.

Method	**Link to EURL SOPs for cattle**
Brucellin Skin Test	Brucellin skin test | EURL (anses.fr)
Brucella culture and genus identification	Brucella culture and genus identification | EURL (anses.fr)
Real-time PCR	Real-time PCR | EURL (anses.fr)
Multiple Locus Variable number tandem repeats Analysis	Multiple Locus Variable number tandem repeats Analysis | EURL (anses.fr)

4.4 Withdrawal and regaining of the status

The Commission Delegated Regulation 2020/689 (Annex IV, part I) describes the situations leading to withdrawal of the status free from infection with *Brucella abortus, B. melitensis* and *B. suis* with (Annex IV, part I, Chapter I, section 4) or without vaccination (Annex IV, part I, Chapter II, section 4) to an establishment keeping bovine, ovine or caprine animals.

The status free from infection with *Brucella abortus, B. melitensis* and *B. suis* of an establishment keeping bovine, ovine or caprine animals must be withdrawn if:

- one or more of the requirements set out in Section 2 (see 3.2) are not fulfilled after the maximum period of time referred to in point (b) of Article 20(3) has lapsed since the status was suspended;

(b) the infection with *Brucella abortus, B. melitensis* and *B. suis* cannot be ruled out in accordance with point 2(b) of Section 3;

(c) a case of infection with *Brucella abortus, B. melitensis* and *B. suis* has been confirmed in a bovine, ovine or caprine animal kept in the establishment; or

(d) it is justified by other needs to control infection with *Brucella abortus, B. melitensis, B. suis*.

4.5 International trade

Commission Delegated Regulation (EU) 2020/692 supplementing Regulation (EU) 2016/429 defines rules for entry into the Union, and the movement and handling after entry of consignments of certain animals, germinal products and products of animal origin (Annex V). The diagnostic methods for Infection with *Brucella abortus, B. melitensis and B. suis* are laid down In Part 1 of Annex I of the Commission Delegated Regulation (EU) 2020/688.

- Serological tests for *Bison* ssp, *Bos* ssp and *Bubalus* ssp:

(a) Buffered Brucella antigen tests;

(b) Complement fixation test (CFT);

(c) Indirect enzyme-linked immunosorbent assay (I-ELISA);

(d) Fluorescence polarisation assay (FPA);

(e) Competitive enzyme-linked immunosorbent assay (C-ELISA).

- Brucellin skin test (BST): not allowed for international trade for *Bison* ssp, *Bos* ssp and *Bubalus* ssp.