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**Changes to legislation:** There are currently no known outstanding effects for the Commission Decision of 10 December 2008 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) (Text with EEA relevance) (2008/984/EC), ANNEX. (See end of Document for details)

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## ANNEX

1. Annex C to Directive 64/432/EEC is replaced by the following:

### ANNEX C

#### BRUCELLOSIS

##### 1. IDENTIFICATION OF THE AGENT

The demonstration by modified acid-fast or immunospecific staining of organisms of *Brucella* morphology in abortion material, vaginal discharges or milk provides presumptive evidence of brucellosis, especially if supported by serological tests. The polymerase chain reaction (PCR) methods provide additional means of detection.

Whenever possible, *Brucella* spp. should be isolated using plain or selective media by culture from uterine discharges, aborted foetuses, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs.

After isolation, the species and biovar shall be identified by phage lysis and/or oxidative metabolism tests, cultural, biochemical and serological criteria. PCR can provide both a complementary and biotyping method based on specific genomic sequences.

The techniques and media used, their standardisation and the interpretation of results must conform to that specified in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition, 2008, Chapter 2.4.3 (bovine brucellosis), Chapter 2.7.2 (caprine and ovine brucellosis) and Chapter 2.8.5 (porcine brucellosis).

##### 2. IMMUNOLOGICAL TESTS

###### 2.1. Standards

2.1.1. The *Brucella abortus* biovar 1 Weybridge strain No 99 or USDA strain 1119-3 must be used for the preparation of all antigens used in the rose Bengal test (RBT), serum agglutination test (SAT), complement fixation test (CFT) and the milk ring test (MRT).

2.1.2. The standard reference serum for the RBT, SAT, CFT and MRT shall be the OIE international reference standard serum (OIEISS) formerly named WHO second international anti-*Brucella abortus* Serum (ISAbS).

2.1.3. The standard reference sera for enzyme-linked immunosorbent assays (ELISAs) shall be:

- the OIEISS,
- the weak positive OIE ELISA standard serum (OIEELISA<sub>WPSS</sub>),
- the strong positive OIE ELISA standard serum (OIEELISA<sub>SPSS</sub>),
- the negative OIE ELISA standard serum (OIEELISA<sub>NSS</sub>).

2.1.4. The standard reference sera for fluorescence polarisation assays (FPAs) shall be:

- the weak positive OIE ELISA standard serum (OIEELISA<sub>WPSS</sub>),
- the strong positive OIE ELISA standard serum (OIEELISA<sub>SPSS</sub>),
- the negative OIE ELISA standard serum (OIEELISA<sub>NSS</sub>).

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2.1.5. The standard sera listed in 2.1.3 and 2.1.4 are available from the Community reference laboratory for brucellosis or the Veterinary Laboratories Agency (VLA), Weybridge, United Kingdom.

2.1.6. The OIEISS, the OIEELISA<sub>WPSS</sub>, the OIEELISA<sub>SPSS</sub> and the OIEELISA<sub>NSS</sub> are international primary standards from which secondary reference national standards serum (working standards) must be established for each test referred to in 2.1.1 in each Member State.

2.2. Enzyme-linked immunosorbent assays (ELISAs) or other binding assays for the detection of bovine brucellosis in serum or milk

2.2.1. Material and reagents

The technique used and the interpretation of results must have been validated in accordance with the principles laid down in Chapter 1.1.4 of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition, 2008, and must include at least laboratory and diagnostic studies.

2.2.2. Standardisation of the test

2.2.2.1. Standardisation of the test procedure for individual serum samples:

- (a) a 1/150 pre-dilution<sup>(1)</sup> of the OIEISS or a 1/2 pre-dilution of the OIEELISA<sub>WPSS</sub> or a 1/16 pre-dilution of the OIEELISA<sub>SPSS</sub> made up in a negative serum (or in a negative pool of sera) must give a positive reaction;
- (b) a 1/600 pre-dilution of the OIEISS or a 1/8 pre-dilution of the OIEELISA<sub>WPSS</sub> or a 1/64 pre-dilution of the OIEELISA<sub>SPSS</sub> made up in a negative serum (or in a negative pool of sera) must give a negative reaction;
- (c) the OIEELISA<sub>NSS</sub> must always give a negative reaction.

2.2.2.2. Standardisation of the test procedure for pooled serum samples:

- (a) a 1/150 pre-dilution of the OIEISS or a 1/2 pre-dilution of the OIEELISA<sub>WPSS</sub> or a 1/16 pre-dilution of the OIEELISA<sub>SPSS</sub> made up in a negative serum (or in a negative pool of sera) and again diluted in negative sera by the number of samples making up the pool must give a positive reaction;
- (b) the OIEELISA<sub>NSS</sub> must always give a negative reaction;
- (c) the test must be adequate to detect evidence of infection in a single animal of the group of animals, of which samples of serum have been pooled.

2.2.2.3. Standardisation of the test procedure for pooled milk or whey samples:

- (a) a 1/1 000 pre-dilution of the OIEISS or a 1/16 pre-dilution of the OIEELISA<sub>WPSS</sub> or a 1/125 pre-dilution of the OIEELISA<sub>SPSS</sub> made up in a negative serum (or in a negative pool of sera) and again diluted 1/10 in negative milk must give a positive reaction;
- (b) the OIEELISA<sub>NSS</sub> diluted 1/10 in negative milk must always give a negative reaction;
- (c) the test must be adequate to detect evidence of infection in a single animal of the group of animals, of which samples of milk or whey have been pooled.

2.2.3. Conditions for use of the ELISAs for diagnosis of bovine brucellosis:

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- 2.2.3.1. Using the calibrating conditions for ELISAs set out in point 2.2.2.1 and 2.2.2.2 on serum samples, the diagnostic sensitivity of ELISA shall be equal or greater than the RBT or CFT taking into account the epidemiological situation under which it is employed.
- 2.2.3.2. Using the calibrating conditions for ELISA set out in point 2.2.2.3 on pooled milk samples, the diagnostic sensitivity of ELISA shall be equal or greater than the MRT taking into account not only the epidemiological situation but also the average and expected extreme husbandry systems.
- 2.2.3.3. Where ELISAs are used for certification purposes in accordance with Article 6(1) or for the establishment and maintenance of a herd status in accordance with Annex A(II)(10), pooling of samples of serum must be carried out in such a way that the test results can be undoubtedly related to the individual animal included in the pool. Any confirmatory test must be carried out on samples of serum taken from individual animals.
- 2.2.3.4. The ELISAs may be used on a sample of milk taken from the milk collected from a farm with at least 30 % of dairy cows in milk. If that method is used, measures must be taken to ensure that the samples taken for examination can be undoubtedly related to the individual animals from which the milk derived. Any confirmatory test must be carried out on samples of serum taken from individual animals.
- 2.3. Complement fixation test (CFT)
  - 2.3.1. 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 4 °C and not frozen.
  - 2.3.2. Serums must be inactivated as follows:
    - bovine serum: 56 to 60 °C for 30 to 50 minutes,
    - porcine serum: 60 °C for 30 to 50 minutes.
  - 2.3.3. In order to carry out the genuine reaction within the test procedure, a complement dose higher than the minimum necessary for total haemolysis shall be used.
  - 2.3.4. In carrying out the complement fixation test, the following controls must be made each time:
    - (a) control of the anti-complementary effect of the serum;
    - (b) control of the antigen;
    - (c) control of sensitised red blood cells;
    - (d) control of the complement;
    - (e) control using a positive serum of sensitivity at the start of the reaction;
    - (f) control of the specificity of the reaction using a negative serum.
  - 2.3.5. Calculation of results

The OIEISS contains 1 000 international CFT units (ICFTU) per ml. If the OIEISS is tested in a given method the result is given as a titre (i.e. highest direct dilution of the OIEISS giving 50 % haemolysis,  $T_{\text{OIEISS}}$ ). The test result for the test serum given as titre ( $T_{\text{TESTSERUM}}$ ) must be

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expressed in ICFTU per ml. In order to convert the expression of a titre into ICFTU, the factor (F) necessary to convert a titre of an unknown test serum ( $T_{\text{TESTSERUM}}$ ) tested by that method into the ICFTU expression can be found from the formula:

$$F = 1\,000 \times 1/T_{\text{OIEISS}}$$

and the content of international CFT units per ml of test serum ( $\text{ICFTU}_{\text{TESTSERUM}}$ ) from the formula:

$$\text{ICFTU}_{\text{TESTSERUM}} = F \times T_{\text{TESTSERUM}}$$

### 2.3.6. Interpretation of results

A serum containing 20 or more ICFTU per ml shall be considered to be positive.

### 2.4. Milk ring test (MRT)

2.4.1. 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 4 °C and not frozen.

2.4.2. The antigen sensitivity 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.

2.4.3. The ring test must be made on samples representing the contents of each milk churn or the content of each bulk tank from the farm.

2.4.4. The milk samples must not have been frozen, heated or subjected to violent shaking.

2.4.5. The reaction must be carried out using one of the following methods:

- on a column of milk of at least 25 mm height and on a volume of milk of 1 ml to which either 0,03 ml or 0,05 ml of one of the standardised stained antigens has been added,
- on a column of milk of at least 25 mm height and on a volume of milk of 2 ml to which 0,05 ml of one of the standardised stained antigens has been added,
- on a volume of milk of 8 ml to which 0,08 ml of one of the standardised stained antigens has been added.

2.4.6. The mixture of milk and antigens must be incubated at 37 °C for 60 minutes, together with positive and negative working standards. A subsequent 16- to 24-hours incubation at 4 °C increases the sensitivity of the test.

2.4.7. Interpretation of results:

- (a) negative reaction: coloured milk, colourless cream;
- (b) positive reaction:
  - identically coloured milk and cream, or
  - colourless milk and coloured cream.

### 2.5. Buffered *Brucella* antigen test (Rose Bengal test (RBT))

2.5.1. The antigen represents a bacterial suspension in buffered *Brucella* antigen diluent at a pH of  $3,65 \pm 0,05$ , stained by the use of rose Bengal dye. The antigen shall be delivered ready for use and must be stored at 4 °C and not frozen.

2.5.2. The antigen shall be 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

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produces a positive reaction with a serum dilution of 1/45 and a negative reaction with a dilution of 1/55.

2.5.3. The RBT shall be carried out in the following manner:

- (a) serum (20-30 µl) is mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter. The mixture is rocked gently for four minutes at ambient temperature, and then observed in a good light for agglutination;
- (b) an automated method may be used but must be at least as sensitive and accurate as the manual method.

2.5.4. Interpretation of results

Any visible reaction shall be considered to be positive, unless there has been excessive drying round the edges.

Positive and negative working standards shall be included in each series of tests.

2.6. Serum agglutination test (SAT)

2.6.1. The antigen represents a bacterial suspension in phenol-saline (NaCl 0,85 % (m/v) and phenol at 0,5 % (v/v)).

Formaldehyde must not be used.

Antigens may be delivered in the concentrated state provided the dilution factor to be used is indicated on the bottle label.

EDTA may be added to the antigen suspension to 5 mM final test dilution to reduce the level of false positives to the serum agglutination test. Subsequently the pH of 7.2 must be readjusted in the antigen suspension.

2.6.2. The OIEISS contains 1 000 international units of agglutination.

2.6.3. The antigen shall be 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 either a 50 % agglutination with a final serum dilution of 1/600 to 1/1 000 or 75 % agglutination with a final serum dilution of 1/500 to 1/750.

It may also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of defined sera.

2.6.4. The test shall be performed either in tubes or in microplates. The mixture of antigen and serum dilutions shall be incubated for 16- to 24-hours at 37 °C.

At least three dilutions must be prepared for each serum. Dilutions of suspect serum must be made in such a way that reading of the reaction at the positivity limit is made in the median tube (or well for the microplate method).

2.6.5. Interpretation of results:

The degree of *Brucella* agglutination in a serum must be expressed in IU per ml.

A serum containing 30 or more IU per ml is considered to be positive.

2.7. Fluorescence polarisation assay (FPA)

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2.7.1. The FPA can be performed in glass tubes or a 96-well plate format. The technique used, its standardisation and the interpretation of results must conform to that specified Chapter 2.4.3 (bovine brucellosis) of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition, 2008.

2.7.2. Standardisation of the test

The FPA shall be standardised so that:

- (a) the OIEELISA<sub>SP</sub>SS and OIEELISA<sub>WP</sub>SS consistently give positive results.
- (b) a 1/8 pre-dilution of the OIEELISA<sub>WP</sub>SS or a 1/64 pre-dilution of the OIEELISA<sub>SP</sub>SS made up in a negative serum (or in a negative pool of sera) always gives a negative reaction;
- (c) the OIEELISA<sub>N</sub>SS always gives a negative reaction.

The following shall be included in each batch of tests: a strong positive, a weak positive, a negative working standard serum (calibrated against the OIE ELISA Standard Sera).

### 3. COMPLEMENTARY TESTS

#### 3.1. Brucellosis skin test (BST)

##### 3.1.1. Conditions for the use of BST

- (a) The brucellosis skin test shall not be used for the purpose of certification for intra-Community trade.
- (b) The brucellosis skin test is one of the most specific tests for the detection of brucellosis in unvaccinated animals; however diagnosis must not be made on the basis of positive intradermal reactions alone.
- (c) Bovine animals, tested with negative result in one of the serological tests defined in this Annex and reacting positively to the BST shall be regarded as infected or suspected to be infected.
- (d) Bovine animals, tested with positive result in one of the serological tests defined in this Annex may be subject to a BST in order to support the interpretation of the serological test results; in particular where in officially brucellosis-free or brucellosis-free bovine herds a cross-reaction with antibodies against other bacteria cannot be excluded.

3.1.2. The test must be carried out by use of a standardised and defined brucellosis allergen preparation that does not contain smooth lipopolysaccharide (LPS) antigen, as this may provoke non-specific inflammatory reactions or interfere with subsequent serological tests.

The requirements for the production of brucellin shall comply with Section C1 of Chapter 2.4.3 of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition, 2008.

##### 3.1.3. Test procedure

3.1.3.1. A volume of 0.1 ml of brucellosis allergen shall be injected intradermally into the caudal fold, the skin of the flank, or the side of the neck.

3.1.3.2. The test shall be read after 48- to 72-hours.

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3.1.3.3. The skin thickness at the injection site shall be measured with vernier callipers before injection and at re-examination.

3.1.3.4. Interpretation of results:

Strong reactions are easily recognised by local swelling and induration.

Skin thickening of 1 to 2 mm shall be considered as positive reaction to the BST.

3.2. Competitive enzyme-linked immunosorbent assay (cELISA)

3.2.1. Conditions for the use of cELISA

The cELISA shall not be used for the purpose of certification for intra-Community trade.

Bovine animals, tested with positive result in one of the other serological tests defined in this Annex may be subject to a cELISA in order to support the interpretation of the other serological test results; in particular where in the officially brucellosis-free or brucellosis-free bovine herds a cross-reaction with antibodies against other bacteria cannot be excluded or to eliminate reactions due to residual antibodies produced in response to vaccination with S19.

3.2.2. Test procedure

The test shall be carried out in accordance with the prescription in Section B(2) of Chapter 2.4.3 of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition, 2008.

2. In Annex C to Directive 64/432/EEC point 4.1 is replaced by the following:

4.1. Tasks and responsibilities

National reference laboratories designated in accordance with Article 6a shall be responsible for:

- (a) the approval of the results of the validation studies demonstrating the reliability of the test method used in the Member State;
- (b) determination of the maximum number of samples to be pooled in ELISA kits used;
- (c) calibration of working standards as referred to in point 2.1.6;
- (d) quality checks of all antigens and ELISA kits batches used in the Member State;
- (e) following recommendations of, and cooperating with the Community reference laboratory for brucellosis.

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- (1) For the purpose of this Annex, dilutions given for making up liquid reagents are expressed as, for example, 1/150 shall mean a 1 in 150 dilution.



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