Council Directive of 26 June 1964 on animal health problems affecting intra-Community trade in bovine animals and swine (64/432/EEC)

[F1[F2ANNEX C

BRUCELLOSIS

Textual Amendments

F1 Substituted by Council Directive 97/12/EC of 17 March 1997 amending and updating Directive 64/432/EEC on health problems affecting intra-Community trade in bovine animals and swine.

F2 Substituted by Commission Regulation (EC) No 535/2002 of 21 March 2002 amending Annex C to Council Directive 64/432/EEC and amending Decision 2000/330/EC.

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.

After isolation, the species and biovar should be identified by phage lysis and/or oxidative metabolism tests, cultural, biochemical and serological criteria.

The techniques and media used, their standardisation and the interpretation of results must conform to that specified in the OIE Manual of Standards for Diagnostic Tests and Vaccines, Fourth Edition, 2000, Chapter 2.3.1 (bovine brucellosis), Chapter 2.4.2 (caprine and ovine brucellosis) and Chapter 2.6.2 (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 is 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 ELISAs are:

— the OIEISS,

— the weak positive OIE ELISA standard serum ($OIEELISA_{WP}SS$),

— the strong positive OIE ELISA standard serum ($OIEELISA_{SP}SS$),

— The negative OIE ELISA standard serum ($OIEELISA_{N}SS$).

2.1.4. The above listed standard sera are available from the Veterinary Laboratories Agency (VLA), Weybridge, United Kingdom.

2.1.5. The OIEISS, the $OIEELISA_{WP}SS$, the $OIEELISA_{SP}SS$ and the $OIEELISA_{N}SS$ are international primary standards from which secondary reference national standards (‘working standards’) must be established for each test 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 1.1.3 of the OIE Manual of Standards for Diagnostic Tests and Vaccines, Fourth Edition, 2000, and should at least include 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[1] of the OIEISS or a 1/2 pre-dilution of the OIEELISA$_{WP}$SS or a 1/16 pre-dilution of the OIEELISA$_{SP}$SS made up in a negative serum (or in a negative pool of sera) should give a positive reaction;

(b) a 1/600 pre-dilution of the OIEISS or a 1/8 pre-dilution of the OIEELISA$_{WP}$SS or a 1/64 pre-dilution of the OIEELISA$_{SP}$SS made up in a negative serum (or in a negative pool of sera) should give a negative reaction;

(c) the OIEELISA$_{N}$SS should 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$_{WP}$SS or a 1/16 pre-dilution of the OIEELISA$_{SP}$SS 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 should give a positive reaction;

(b) the OIEELISA$_{N}$SS should 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 1/1000 pre-dilution of the OIEISS or a 1/16 pre-dilution of the OIEELISA$_{WP}$SS or a 1/125 pre-dilution of the OIEELISA$_{SP}$SS made up in a negative serum (or in a negative pool of sera) and again diluted 1/10 in negative milk should give a positive reaction;

(b) the OIEELISA$_{N}$SS diluted 1/10 in negative milk should 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:

2.2.3.1. Using the abovementioned calibrating conditions for ELISAs 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 abovementioned calibrating conditions for ELISA 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 this 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 ^{o}C and not frozen.

2.3.2. Serums must be inactivated as follows:

— bovine serum: 56 to 60 ^{o}C for 30 to 50 minutes,

— porcine serum: 60 ^{o}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 should 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 (T_{OIEISS}). The test result for the test serum given as titre ($T_{TESTSERUM}$) must be 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 ($T_{TESTSERUM}$) tested by that method into the ICFTU expression can be found from the formula:

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

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

$$ICFTU_{TESTSERUM} = F \times T_{TESTSERUM}$$

2.3.6. Interpretation of results

A serum containing 20 or more ICFTU per ml is 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 ^{o}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 should 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 ^{o}C for 60 minutes, together with positive and negative working standards. A subsequent 16 to 24 hour incubation at 4 ^{o}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. Rose bengal plate Test (RBT)

2.5.1. 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 4 ^{o}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 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 4 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 is considered to be positive, unless there has been excessive drying round the edges.

Positive and negative working standards should 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 a 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 is performed either in tubes or in microplates. The mixture of antigen and serum dilutions should be incubated for 16 to 24 hours at 37 ^{o}C.

At least three dilutions must be prepared for each serum. Dilutions of suspect serum must be made in such a way that the 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.

3. COMPLEMENTORY 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 should 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.

(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 brucellosis free or officially free 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.

One of such preparation is Brucellin INRA prepared from a non smooth strain of *B. melitensis*. The requirements for its production are detailed in Section B2 of Chapter 2.4.2. of the OIE Manual of Standards for Diagnostic Tests and Vaccines, Fourth Edition, 2000.

3.1.3. Test procedure

3.1.3.1. A volume of 0,1 ml of brucellosis allergen is injected intradermally into the caudal fold, the skin of the flank, or the side of the neck.

3.1.3.2. The test is read after 48-72 hours.

3.1.3.3. The skin thickness at the injection site is 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 1,5 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

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

(b) The cELISA has shown to have a higher specificity than for example the indirect ELISA and may therefore be used in order to support the interpretation of the serological test results.

3.2.2. Test procedure

The test shall be carried out in accordance with the prescription in the OIE Manual of Standards for Diagnostic Tests and Vaccines, Fourth Edition, 2000, Chapter 2.3.1(2)(a).

4. NATIONAL REFERENCE LABORATORIES

4.1. Tasks and responsibilities

National reference laboratories 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 the standard secondary reference national standard sera (‘working standards’) against the primary international standard serum referred to in paragraph 2.1;

(d) quality checks of all antigens and ELISA kits batches used in the Member State;

(e) cooperation within the European Union Network of National Reference Laboratories for Brucellosis.

[F3 4.2.

LIST OF NATIONAL REFERENCE LABORATORIES

AT	AGES: Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH — Institut für veterinärmedizinische Untersuchungen Mödling (Austrian Agency for Health and Consumer Protection-Institute for veterinary investigations Mödling) Robert Koch-Gasse 17 A-2340 Mödling Tel.: +43 (0) 505 55-38112 Fax: +43 (0) 505 55-38108 E-mail: vetmed.moedling@ages.at
BE	CODA — CERVA — VAR Veterinary and Agrochemical Research Centre Groeselenberg 99 B-1180 Brussels
[F4 BG	Национален диагностичен научноизследователски ветеринарномедицински институт Проф. д-р Георги Павлов, Национална референтна лаборатория Бруцелоза по животните, бул. Пенчо Славейков 15, София 1606 (National Diagnostic Veterinary Research Institute Prof. Dr. Georgi Pavlov, National Reference Laboratory for Brucellosis, 15, Pencho Slaveykov Blvd., 1606 Sofia)**]**
CY	State Veterinary Laboratory Veterinary Services 1417 Athalassa Nicosia
CZ	Státní veterinární ústav Olomouc Jakoubka ze Stříbra 1 779 00 Olomouc
[F5 DE	Friedrich-Loeffler-Institut Bundesforschungsinstitut für Tiergesundheit Boddenblick 5a 17493 Greifswald — Insel Riems Tel. (49-38351) 7-0 Fax (49-38351) 7-219

	E-Mail: poststelle@fli.bund.de
DK	National Veterinary Institute, Technical University of Denmark Bülowsvej 27 DK-1790 Copenhagen V**]**
EE	Veterinaar- ja Toidulaboratoorium Kreutzwaldi 30, 51006 Tartu, Estonia Tel.: +372 7 386 100 Faks: +372 7 386 102 E-post: info@vetlab.ee
ES	Laboratorio Central de Sanidad Animal de Santa Fe Camino del Jau s/n Santa Fe 18320 (Granada) Tel.: 34 958 440 375/440 400 Fax: 34 958 441 200 Fulgencio Garrido Abellán E-mail: clvgr@mapya.es
FI	Finnish Food Safety Authority Animal Diseases and Food Safety Research Mustialankatu 3 FI-00790 Helsinki, Finland E-mail: info@evira.fi Tel.: +358 20 772 003 (exchange) Fax: +358 20 772 4350
[[F5]FR	Laboratoire d'études et de recherches en pathologie animale et zoonoses AFSSA-LERPAZ 23, avenue du Général-de-Gaulle F-94703 Maisons-Alfort Cedex**]**
GB	Veterinary Laboratories Agency New Haw, Addlestone, Weybridge Surrey KT15 3NB, UK Tel. (44-1932) 341111 Fax (44-1932) 347046 Immunodiagnostics Department Veterinary Sciences Division Stoney Road Stormont Belfast BT4 3SD, UK
GR	Hellenic Ministry of Rural Development and Food National Veterinary Laboratory of Larisa 6o Km, National Highway Larisa-Trikala Tel.: + 30 2410 617 980-617 981 Fax: + 30 2410 617982
[[F5]HU	Mezőgazdasági Szakigazgatási Hivatal Központ, Állat-egészségügyi Diagnosztikai Igazgatóság

	Central Agricultural Office, Veterinary Diagnostic Directorate Address: 1149 Budapest, Tábornok u. 2. Mailing Address: 1581 Budapest, 146. Pf. 2. Tel.: +36 1 460-6300 Fax: +36 1 252-5177 E-mail: titkarsag@oai.hu**]**
IE	The Blood Testing Laboratory Department of Agriculture and Food Model Farm Road Cork Co. Cork
IT	Centro di Referenza Nazionale per le brucellosi c/o Istituto zooprofilattico sperimentale dell' Abruzzo e del Molise Via Campo Boario I- 64100 Teramo
LT	Nacionalinė veterinarijos laboratorija, J. Kairiūkščio g. 10, LT-2021 Vilnius
LU	CODA — CERVA — VAR Veterinary and Agrochemical Research Centre Groeselenberg 99 B-1180 Brussels
LV	Nacionālais diagnostikas centrs (National Diagnostic Centre) Lejupes iela 3, Rīga, LV-1076 Tel.: +371 7620526 Fax: +371 7620434 E-mail: ndc@ndc.gov.lv
MT	—
NL	Centraal Instituut voor DierziekteControle CIDC-Lelystad Hoofdvestiging: Houtribweg 39 Nevenvestiging: Edelhertweg 15 Postbus 2004 8203 AA Lelystad
PL	Laboratory Departament of Microbiology Państwowy Instytut Weterynaryjny – Państwowy Instytut Badawczy, Al. Partyzantów 57, 24-100 Puławy Tel.: +48.81.886 30 51 Fax: +48.81.886 25 95 E-mail: sekretariat@piwet.pulawy.pl
PT	Laboratório Nacional de Investigação Veterinária (LNIV) Estrada de Benfica, 701

	P-1549-011 Lisboa
[F4RO	Institutul de Diagnostic şi Sănătate Animală Strada Dr. Staicovici nr. 63, sector 5 codul 050557, Bucureşti]
SE	Statens Veterinärmedicinska Anstalt SE-751 89 Uppsala
SI	Univerza v Ljubljani Veterinarska fakulteta Nacionalni veterinarski inštitut Gerbičeva 60, SI-1000 Ljubljana
SK	Štátny veterinárny ústav Pod dráhami 918 SK-960 86 Zvolen]]]

Textual Amendments

F3 Substituted by Commission Decision of 5 December 2006 amending Council Directives 64/432/EEC, 90/539/EEC, 92/35/EEC, 92/119/EEC, 93/53/EEC, 95/70/EC, 2000/75/EC, 2001/89/EC, 2002/60/EC and Decision 2001/618/EC as regards lists of national reference laboratories and State institutes (notified under document number C(2006) 5856) (Text with EEA relevance) (2006/911/EC).

F4 Inserted by Council Directive 2006/104/EC of 20 November 2006 adapting certain Directives in the field of agriculture (veterinary and phytosanitary legislation), by reason of the accession of Bulgaria and Romania.

F5 Substituted by Commission Decision of 7 November 2007 amending Council Directives 64/432/EEC, 90/539/EEC, 92/35/EEC, 92/119/EEC, 93/53/EEC, 95/70/EC, 2000/75/EC, 2001/89/EC, 2002/60/EC, and Decisions 2001/618/EC and 2004/233/EC as regards lists of national reference laboratories and State institutes (notified under document number C(2007) 5311) (Text with EEA relevance) (2007/729/EC).

(1) **[**[F1]**[**[F2]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.**]]**

Textual Amendments

F1 Substituted by Council Directive 97/12/EC of 17 March 1997 amending and updating Directive 64/432/EEC on health problems affecting intra-Community trade in bovine animals and swine.

F2 Substituted by Commission Regulation (EC) No 535/2002 of 21 March 2002 amending Annex C to Council Directive 64/432/EEC and amending Decision 2000/330/EC.