II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DECISION

of 25 February 1991

establishing the protocols for the standardization of materials and procedures for veterinary tests and the conditions for the approval of markets in connection with the import of domestic animals of the bovine and porcine species from third countries

(91/189/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community.

Having regard to Council Directive 72/462/EEC of 12 December 1972 on health and veterinary inspection problems related to the importation of domestic animals of the bovine and porcine species and of fresh meat from third countries [1], as most recently modified by Directive 91/69/EEC [2] and in particular Article 8 (1) thereof,

Whereas Directive 72/462/EEC authorizes the importation of domestic animals of the bovine and porcine species from third countries which are included in the list annexed to Council Decision 79/542/EEC [3], as last modified by Decision 90/485/EEC [4];

Whereas the animal health conditions to be applied in respect of the import of live animals from a third country must be established in accordance with the animal health situation in that country;

Whereas although these animal health conditions may vary from country to country, the protocols for veterinary tests and the conditions for the approval of markets in third countries for trade in animals intended for export to the Community are applicable to all third countries;

Whereas in order to simplify the Decisions regarding the animal health conditions to be applied in respect of the import of domestic animals of the bovine and porcine species from third countries it would appear useful to refer to a single common Decision containing the protocols for veterinary tests and the conditions for the approval of markets;

Whereas a Decision setting out the protocols for veterinary tests and the conditions for the approval of markets in third countries shall only apply in respect of a third country for which a Decision specifically related to the animal health conditions in that country has been adopted;

Whereas the measures provided for in this Decision are in accordance with the opinion of the Standing Veterinary Committee,

HAS ADOPTED THIS DECISION:

Article 1

1. This Decision provides for the establishment of protocols for veterinary tests and conditions for the

(1) OJ No L 302, 31. 12. 1972, p. 28.
(2) OJ No L 46, 19. 2. 1991, p. 37.
(3) OJ No L 146, 14. 6. 1979, p. 15.
(4) OJ No L 276, 27. 9. 1990, p. 46.

approval of markets in third countries for trade in animals intended for export to the Community in relation to the import of live animals of the bovine and porcine species from third countries included in the list annexed to Decision 79/542/EEC.

2. The requirements set out in Annexes I and II to this Decision shall apply only to third countries in respect of which Decisions have been adopted setting out the animal health requirements in each case in the terms of Article 8 of Directive 72/462/EEC.

Article 2

This Decision is addressed to the Member States.

Done at Brussels, 25 February 1991.

For the Commission

Ray MAC SHARRY

Member of the Commission

ANNEX I

PROTOCOLS FOR THE STANDARDIZATION OF MATERIALS AND TESTING PROCEDURES

CHAPTER I

Bovine Animals

1. *Tuberculosis*

The single intradermal tuberculin test using bovine tuberculin shall be carried out according to Annex B to Directive 64/432/EEC of 26 June 1964 [1] on animal health problems affecting intra-Community trade in bovine animals and swine.

2. *Brucellosis*

The serum agglutination test and complement fixation test shall be carried out according to paragraphs A and B of Annex C to Directive 64/432/EEC.

3. *Enzootic Bovine Leukosis*

The agar gel immuno-diffusion test shall be carried out according to Annex G to Council Directive 64/432/EEC.

4. *Bluetongue*

A. The blocking or competitive Elisa test shall be carried out according to the following protocol:

The competitive Elisa using monoclonal antibody 3-17-A3 is capable of detecting antibodies to all known serotypes of bluetongue virus (BTV).

The principle of the test is the interruption of the reaction between BTV antigen and a group-specific monoclonal antibody (3-17-A3) by the addition of test serum dilutions. Antibodies to BTV present in the test serum block the reactivity of the monoclonal antibody (Mab) and result in a reduction in the expected colour development on addition of enzyme substrate.

Material and Reagents:

1. Flat-bottomed microtitre plates.
2. Antigen: prepared as described below.
3. Blocking buffer: 5 % (w/v) 'Marvel' dried milk powder, 0,1 % (v/v) Tween-20 (supplied as polyoxyethylene sorbiton monolaurate syrup) in PBS.
4. Monoclonal antibody: 3-17-A3 (supplied as hybridoma tissue-culture supernatant) stored at − 20 °C or freeze-dried, diluted 1/50 with blocking buffer before use, directed against the group-specific polypeptide p7.
5. Conjugate: rabbit anti-mouse globulin (adsorbed and eluted) conjugated to horseradish peroxidase and kept in the dark at 4° C.
6. Substrate and chromogen: 0,2 gm of orthophenylene diamine (OPD) dissolved in a buffer consisting of 2,553 g of citric acid and 4,574 g of di-sodium hydrogenorthophosphate made up to 500 ml with distilled water, divided into 25 ml aliquots and kept in the dark at − 20° C, with 12 µl/25 ml of hydrogen peroxide (30 % w/v) added immediately before use.

Handle OPD with care — wear rubber gloves — suspected mutagen.

7. 1 Molar sulphuric acid: 26,6 ml of acid added to 473,4 ml of distilled water.

Remember — always add acid to water, never water to acid.

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[1] OJ No L 121, 20. 7. 1964, p. 1977/64.

8. Orbital shaker.

9. Elisa plate reader (the test may be read visually).

Test format

	H	G	F	E	D	C	B	A	
Blank			Antigen + conjugate						1
Mab-control		Antigen + Mab + conjugate							2
+ve control	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	3
Test sera	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	4
									5
									6
									7
									8
									9
									10
									11
									12

Test protocol:

Blank control:

row 1 A — H is a blank control consisting of BTV antigen and conjugate. This may be used to blank the Elisa reader.

Mab control:

row 2 A — H is the monoclonal antibody control and consists of BTV antigen, monoclonal antibody and conjugate. This represents a negative control. The mean of the optical density readings from this control row represents the 0 % inhibition value.

Positive control:

row 3 A — H is the positive control. This consists of BTV antigen, BTV positive antiserum dilutions, Mab and conjugate. This in included as an indicator that the test is functioning properly and similar levels of inhibition should be obtained from test to test.

Test sera:

in the test format shown above, 18 sera can be tested over a dilution rate of 1/2, 1/4, 1/8 and 1/16. This will give some indication of the titre of antibody in the test sera. The dilution range could be extended further to obtain serum dilution end-point titres. Alternatively, for large-scale serological surveys, sera could be tested at a single dilution (1/4) or two dilutions (1/2 and 1/4) as a rapid screening test.

Procedure:

1. Dilute BTV antigen to pre-titrated concentration in PBS, sonicate briefly to disperse aggregated virus (if sonicator is not available, pipette vigorously) and add 50 µl to all wells of the Elisa plate. Tap sides of plate to disperse antigen.

2. Incubate at 37 °C for 60 minutes on an orbital shaker. Wash plates three times by flooding and emptying the wells with unsterile PBS and blot dry on absorbent paper.

2. Add 50 µl per well of blocking buffer. Add test sera and positive serum to the appropriate wells and dilute across the plate using a multichannel pipette. Do not add sera to the blank control or the Mab control.

4. Immediately after the addition of the test sera, dilute Mab in blocking buffer (to pre-titrated dilution) and add 50 µl to all wells of the plate except for the blank control.

5. Incubate at 37 °C for 60 minutes on an orbital shaker. Wash three times with PBS and blot dry.

6. Dilute rabbit anti-mouse concentrate to 1/5 000 in blocking buffer and add 50 µl to all wells of the plate.

7. Incubate at 37 °C for 60 minutes on an orbital shaker. Wash three times with PBS and blot dry.

8. Thaw the OPD and immediately before use add 12 µl of 30 % hydrogen peroxide to each 25 ml of OPD. Add 50 µl to all wells of the plate. Allow colour to develop for approximately 10 minutes and stop the reaction with 1 M sulphuric acid (50 µl per well). Colour should develop in the Mab control wells and in those wells containing sera with no antibody to BTV.

9. Examine and record the plates either visually or using a spectrophotometric reader.

Analysis of results:

Calculate the mean OD reading from the Mab controls. This represents the 0 % inhibition value. Optical density readings from the test sera are expressed as percentage inhibition values using the following formula:

$$\text{Percentage inhibition value} = 100 \frac{\text{OD in the presence of test serum}}{\text{OD in the absence of test serum}} \times 100.$$

Inhibition values greater than 40 % at a serum dilution of 1/4 are considered positive. Visual reading is possible as 40 % inhibition is the lowest value easily discernible by eye.

Preparation of BTV Elisa antigen:

1. Wash 10 roux of confluent BHK-21 cells three times with serum-free Eagle's medium and infect with bluetongue virus serotype 1 in serum-free Eagle's medium.

2. Incubate at 37 °C and examine daily for cytopathic effect (cpe).

3. When cpe is evident in 80 to 90 % of the cell sheet of each roux, harvest the virus by shaking any still-attached cells from the glass.

4. Centrifuge at 2 000 to 3 000 rpm to pellet the cells.

5. Discard the supernatant and re-suspend the cells in approximately 30 ml of PBS containing 1 % 'Sarkosyl' and 2 ml phenolmethylsulphonyl fluoride (lysis buffer). This may cause the cells to form a gel and more lysis buffer may be added to reduce this effect.

   NB: phenylmethylsulphonyl fluoride is harmful — handle with extreme caution.

6. Disrupt the cells for 60 seconds using an ultrasonic probe at an amplitude of 30 microns.

7. Centrifuge at 10 000 rpm for 10 minutes.

8. Store the supernatant at +4 °C and re-suspend the remaining cell pellet in 10 to 20 ml of lysis buffer.

9. Sonicate and clarify, storing the supernatant at each stage, a total of three times.

10. Pool the supernatants and centrifuge at 24 000 rpm for 120 minutes at +4 °C over a 5 ml cushion of 40 % sucrose (w/v in PBS) using 30 ml Beckmann centrifuge tubes and an SW 28 rotor.

11. Discard the supernatant, drain the tubes thoroughly and re-suspend the pellet in PBS by sonication. Store the antigen in aliquots at −70 °C.

Titration of BTV Elisa antigen:

Bluetongue Elisa antigen is titrated by the indirect Elisa. Twofold dilutions of antigen are titrated against a constant dilution (1/50) f monoclonal antibody 3-17-A3. The protocol is as follows:

Procedure:

1. Dilute BTV antigen in PBS across the microtitre plate in a twofold dilution series (50 µl/well) using a multichannel pipette.

2. Incubate for one hour at 37 °C on an orbital shaker.

3. Wash plates three times with PBS.

4. Add 50 µl of monoclonal antibody 3-17-A3 (diluted 1/50) to each well of the microtitre plate.

5. Incubate for one hour at 37 °C on an orbital shaker.

6. Wash plates three times with PBS.

7. Add 50 µl of rabbit anti-mouse globulin conjugated to horseradish peroxidase, diluted to a pre-titrated optimal concentration, to each well of the microtitre plate.

8. Incubate for one hour at 37 °C on an orbital shaker.

9. Add substrate and chromogen as described previously. Stop the reaction after 10 minutes by the addition of 1 Molar sulphuric acid (50 µl/well).

In the competitive assay, the monoclonal antibody must be in excess, therefore a dilution of antigen is chosen which falls on the titration curve (not on the plateau region) which gives approximately 0,8 OD after 10 minutes.

B. The agar gel immuno-diffusion test shall be carried out according to the following protocol:

Antigen:

Precipitating antigen is prepared in any cell culture system that supports the rapid multiplication of a reference strain of bluetongue virus. BHK or Vero cells are recommended. Antigen is present in the supernatant fluid at the end of virus growth but requires 50 to 100-fold concentration to be effective. This may be achieved by any standard protein concentration procedure; virus in the antigen may be inactivated by the addition of 0,3 % (v/v) beta-propiolactone.

Known positive control serum:

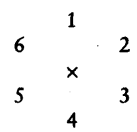
Using the international reference serum and antigen a national standard serum is produced, standardized for optimal proportion against the international reference serum, freeze-dried and used as the known control serum in each test.

Test serum

Procedure:

1 % agarose prepared in borate or sodium barbitol buffer, pH 8,5 to 9,0, is poured into a petri dish to a minimum depth of 3,0 mm.

A test pattern of seven moisture-free wells, each 5,0 mm in diameter, is cut in the agar. The pattern consists of one centre well and six wells arranged round it in a circle of radius 3 mm.



The central well is filled with the standard antigen. Peripheral wells 2, 4 and 6 are filled with known positive serum, wells 1, 3 and 5 are filled with test sera.

The system is incubated for up to 72 hours at room temperature in a closed humid chamber.

Interpretation:

A test serum is positive if it forms a specific precipitin line with the antigen and forms a complete line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and it does not bend the line of the control serum. Petri dishes should be examined against a dark background and using indirect illumination.

5. *Epizootic haemorrhagic disease*

The agar gel immuno-diffusion test shall be carried out according to the following protocol:

Antigen:

Precipitating antigen is prepared in any cell culture system that supports the rapid multiplication of the appropriate serotype(s) of epizootic haemorrhagic disease virus. BHK or Vero cells are recommended. Antigen is present in the supernatant fluid at the end of virus growth but requires 50 to 100-fold concentration to be effective. This may be achieved by any standard protein concentration procedure; virus in the antigen may be inactivated by the addition of 0,3 % (v/v) beta-propiolactone.

Known positive control serum:

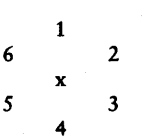
Using the international reference serum and antigen a national standard serum is produced, standardized for optimal proportion against the international reference serum, freeze-dried and used as the known control serum in each test.

Test serum

Procedure:

1 % agarose prepared in borate or sodium barbitol buffer, pH 8,5 to 9,0, is poured into a petri dish to a minimum depth of 3,0 mm.

A test pattern of seven moisture-free wells, each 5,0 mm in diameter, is cut in the agar. The pattern consists of one centre well and six wells arranged round it in a circle of radius 3 mm.



The central well is filled with the standard antigen. Peripheral wells 2, 4 and 6 are filled with known postive serum, wells 1, 3 and 5 are filled with test sera.

The system is incubated for up to 72 hours at room temperature in a closed humid chamber.

Interpretation:

A test serum is positive if it forms a specific precipitin line with the antigen and forms a complete line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and it does not bend the line of the control serum. Petri dishes should be examined against a dark background and using indirect illumination.

6. *Leptospirosi*

The microscopic agglutination test shall be carried out according to the following protocol:

Cultures:

are maintained in Korthof's or EMJH medium at 30 °C.

Antigen:

contains 2×10^8 organisms per ml of culture medium.

Procedure:

equal amounts of antigen and serum are mixed in flat-bottomed microtitre plates and incubated at 30 °C for two hours or 37 °C for one to one and a half hours. The test is read by low-power dark-field illumination using a magnification between 60x and 100x.

Interpretation:

a negative result is less than 50 % agglutination at a dilution of 1/50.

7. *Infectious bovine rhino-tracheitis/infectious pustuiar vulvo-vaginitis*

The serum neutralization test shall be carried out according to the following protocol:

Serum:

All sera are heat-inactivated at 56 °C for 30 minutes before use.

Procedure:

The constant virus-varying serum neutralization test on microtitre plates employs MDBK or other susceptible cells. The Colorado, Oxford or any other reference strain of the virus is used at 100 $TCID_{50}$ per 0,025 ml; inactivated undiluted serum samples are mixed with an equal volume (0,025 ml) of virus supension. The virus/serum mixtures are incubated for two hours at 37 °C in the microtitre plates before the MDBK cells are added. Cells are used at a concentration which forms a complete monolayer after 24 hours.

Controls:

virus infectivity assay,
serum toxicity controls,
uninoculated cell culture controls,
reference antisera.

Interpretation:

The results of the neutralization test and the titre of the virus used in the test are recorded after three to six days incubation at 37 °C. Serum titres are considered negative if there is no neutralization at a dilution of 1/2 (undiluted serum).

8. *Bovine virus diarrhoea (BVD)*

A. The virus isolation test shall be carried out according to the following protocol:

Test material and detection methods:

Serum, buffy coat or blood clot suspensions from freshly-collected blood samples, which have not been heat-inactivated, from cattle more than six months old are used. An immunological staining procedure such as the fluorescent antibody technique (FA) or an enzyme-linked antibody test e. g. immunoperoxidase (IPX) are used to detect BVD virus in inocuiated cultures.

Test reagents:

For the FA test an FITC-conjugated specific BVD antiserum is required. For the IPX test an unconjugated specific anti-BVD serum of bovine origin, a peroxidase conjugated anti-bovine antiserum and suitable enzyme substrate (such as 3-amino-9-ethylcarbazole) are required. An anti-BVD serum raised in a species other than bovine may be used provided that the peroxidase conjugate is directed against serum globulins of that species. All antiviral sera used must be of proven specificity and have broad reactivity.

Procedure:

The test uses susceptible cells such as bovine turbinate, calf kidney or calf testis free of endogenous BVD virus.

Serum samples — the test serum and tissue culture cells are placed in coverslip cultures or in wells of microtitre plates or other containers so that the final dilution of serum is 10 %.

Buffy coat and blood clot — the samples are added to confluent cell monolayer cultures for one hour at 37 °C, then the cultures are washed and overlaid with culture medium containing 10 % BVD-free foetal calf serum.

The cultures are then incubated at 37 °C, fixed and stained by either FA or IPX methods.

Controls:

positive BVD virus control,
negative control with no added virus.

Interpretation:'

The Fa test is stained after four to six days' incubation and read using ultra-violet light. Fluorescence in cells incubated with the test sample is interpreted as a positive result.

The IPX test ist stained after four days' incubation and read by light microscopy. Dark brown staining in the cytoplasm of some of the cells is interpreted as a positive result.

B. The serum neutralization test shall be carried out according to the following protocol:

Serum:

All sera are heat-inactivated at 56 °C for 30 minutes before use.

Procedure:

The constant virus-varying serum neutralization test on microtitre plates employs suitable serially propagated susceptible bovine cells (e. g. bovine turbinate cells as described by McClurkin and others, 1974, Arch. ges. Virusforsch., 45, 285 to 289).

It is essential that all reagents and cells should be free from contaminating adventitious noncytopathic BVD/MD virus.

The test virus, which may be any suitable cytopathic reference strain (such as the NADL strain) is used at a concentration of 100 median cell culture infectious doses per 0,025 ml. Dilutions of inactivated sera are mixed with equal volumes of virus suspension (0,025 ml) and the virus-serum mixtures are incubated for one hour at 37 °C before similar volumes of cell suspension are added. The cells are used at a concentration which will form a complete monolayer within two days.

Controls:

virus infectivity assay,
serum toxicity controls,
uninoculated cell culture controls,
reference antisera.

Interpretation:

With NADL strain virus the optimum time for reading is after five days incubation at 37 °C.

A median neutralization titre of one-tenth is considered to be indicative of an immune response to past acute infection.

9. *Milk analysis for mastitis*

Milk analysis shall be carried out according to Annex D to Directive 64/432/EEC.

10. *Foot-and-mouth disease (FMD)*

A. Collecting oesophageal/pharyngeal samples and testing shall be carried out according to the following protocol:

Reagents:

Prior to sampling, transport medium is prepared. Two ml volumes are dispensed in as many containers as there are animals to be sampled. The containers used should withstand freezing over solid CO_2 or liquid nitrogen.

Samples are obtained by the use of a specially-designed sputum collector or 'probang'.

To obtain a sample the probang cup is passed through the mouth, over the dorsum of the tongue and down into the upper part of the oesophagus. Attempts are made to scrape the surface epithelium of the upper oesophagus and pharynx by movements directed laterally and dorsally. The probang is then withdrawn, preferably after the animal has swallowed. The cup should be full and contain a mixture of mucus, saliva, oesophageal fluid and cellular debris. Care should be taken to ensure that each specimen contains some visible cellular material. Very rough handling which causes bleeding should be avoided.

Samples from some animals may be heavily contaminated with ruminal contents. Such samples should be discarded and the mouth of the animal flushed with water, or preferably physiological saline, before repeat sampling.

Treatment of samples:

Each sample collected in the probang cup is examined for quality and 2 ml added to an equal volume of transport medium in a container which can withstand freezing. The containers are tightly closed, sealed, disinfected and labelled. The samples are kept cool (+ 4 °C) and examined within three to four hours or placed over dry ice (– 69 °C) or liquid nitrogen and kept frozen until examined.

Between animals the probang is disinfected and washed in three changes of clean water.

Testing for FMD virus:

Samples are inoculated into cultures of primary bovine thyroid cell cultures using at least three tubes per sample. Other susceptible cells e. g. primary bovine or porcine kidney cells can be used but it should be kept in mind that for some strains of FMD virus they are less sensitive. The tubes are incubated at 37 °C on a roller apparatus and examined daily for 48 hours for the presence of a cytopathic effect (CPE). If negative, cultures are blind passaged onto new cultures and re-examined for 48 hours. The specificity of any CPE must be confirmed.

Recommended transport media:

1. 0,08M phosphate buffer pH 7,2 containing 0,01 % bovine serum albumin, 0,002 % phenol red and antibiotics.

2. Tissue culture medium (e.g. Eagle's MEM) containing 0,04M Hepes buffer, 0,01 % bovine serum albumin and antibiotics, pH 7,2.

Antibiotics (per ml final) should be added to the transport medium e. g.:

— penicillin	1 000 IU,
— neomycin sulphate	100 IU,
— polymyxin B sulphate	50 IU,
— mycostatin	100 IU.

B. The virus neutralization test shall be carried out according to the following protocol:

Reagents:

Stock FMDV antigen is prepared in cell cultures or on cattle tongues and stored at – 70 °C or less or at – 20 °C after the addition of 50 % glycerol. This is the stock antigen. FMDV is stable under these conditions and titres vary little over a period of months.

Procedure:

The test is carried out in flat-bottomed tissue culture grade microtitre plates using susceptible cells such as IB-RS-2, BHK-21 or calf kidney cells.

Sera for the test are diluted 1/4 in serum-free cell culture medium with the addition of 100 IU/ml neomycin or other suitable antibiotics. Sera are inactivated at 56 °C for 30 minutes and 0,05 ml amounts are used to prepare a twofold series on microtitre plates using 0,05 ml diluting loops. Pre-titrated virus also diluted in serum-free culture medium and containing 100 $TCID_{50}$/0,05 ml is then added to each well. Following incubation at 37 °C for one hour to allow neutralization to take place, 0,05 ml of suspension cells containing 0,5 to 1,0 × 10^6 cells per 1 ml in cell culture medium containing serum free of FMD antibody is added to each well and the plates are sealed.

Plates are incubated at 37 °C. Monolayers are normally confluent within 24 hours. CPE is usually sufficiently advanced at 48 hours for a *microscopic* reading of the test. At this time a final *microscopic* reading may be made or the plates may be fixed and stained for *macroscopic* reading, for instance using 10 % formol-saline and 0,05 % methylene blue.

Controls:

Controls in each test include homogous antiserum of known titre, a cell control, a serum toxicity control, a medium control, and a virus titration from which the actual amount of virus in the test is calculated.

Interpretation:

Wells with evidence of CPE are considered to be infected and neutralization titres are expressed as the reciprocal of the final dilution of serum present in the serum/virus mixtures at the 50 % end point estimated according to the Spearman-Karber method. (Karber, G., 1931, *Archiv für Experimentelle Pathologie und Pharmokologie,* 162, 480.)

Tests are considered to be valid when the actual amount of virus used per well in the test is between $10^{1,5}$ and $10^{2,5}$ $TCID_{50}$ and when the titre of the reference serum is within twofold of its expected titre, estimated from the mode of previous titrations. When the controls are outside these limits the tests are repeated.

An end point titre of 1/11 or less is taken as negative.

C. The detection and quantitation of antibody by Elisa shall be carried out according to the following protocol:

Reagents:

Rabbit antisera to 146S antigen of seven types of foot-and-mouth disease virus (FMDV) used at a predetermined optimum concentration in carbonate/bicarbonate buffer, pH 9,6.

Antigens are prepared from selected strains of virus grown on monolayers of BHK-21 cells. The unpurified supernatants are used and pretitrated according to the protocol but without serum, to give a dilution which after the addition of an equal volume of PBST (phosphate buffered saline containing 0,05 % Tween-20 and phenol red indicator) would give an optical density reading of between 1,2 and 1,5. The viruses can be used inactivated. PBST is used as a diluent. Guinea-pig antisera are prepared by inoculating guinea-pigs with 146S antigen of each serotype. A predetermined optimum concentration is prepared in PBST containing 10 % normal bovine serum and 5 % normal rabbit serum.

Rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase is used at a predetermined optimum concentration in PBST containing 10 % normal bovine serum and 5 % normal rabbit serum.

Test sera are diluted in PBST.

Procedure:

1. Elisa plates are coated with 50 µl of rabbit antiviral sera overnight in a humidity chamber at room temperature.

2. Fifty microlitres of a duplicate, twofold series of each test serum starting at 1/4 are prepared in U-bottomed multiwell plates (carrier plates). Fifty microlitres of a constant dose of antigen are added to each well and the mixtures are left overnight at 4 °C. The addition of the antigen reduces the starting serum dilution to 1/8.

3. The Elisa plates are washed five times with PBST.

4. Fifty microlitres of serum/antigen mixtures are then transferred from the carrier plates to the rabbit-serum-coated Elisa plates and incubated at 37 °C for one hour on a rotary shaker.

5. After washing, 50 µl of guinea-pig antiserum to the antigen used in point 4 is added to each well. The plates are incubated at 37 °C for one hour on a rotary shaker.

6. The plates are washed and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase is added to each well. The plates are incubated at 37 °C for one hour on a rotary shaker.

7. The plates are washed and 50 µl of orthophenylene diamine containing 0,05 % H_2O_2 (30 %) w/v is added to each well.

8. The reaction is stopped after 15 minutes with 1,25M H_2SO_4.

The plates are read spectrophotometrically at 492 nm on an Elisa reader linked to a microcomputer.

Controls:

For each antigen used 40 wells contain no serum but contain antigen diluted in PBST.

A duplicated twofold dilution series of homologous bovine reference antiserum.

A duplicate twofold dilution series of negative bovine serum.

Interpretation:

Antibody titres are expressed as the final dilution of tests serum giving 50 % of the mean OD value recorded in the virus control wells where test serum is absent. Titres in excess of 1/40 are considered positive.

References:

Hamblin C, Barnett ITR and Hedger RS (1986) 'A new enzyme-linked immunosorbent assay (Elisa) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of Elisa.' *Journal of Immunological Methods,* 93, 115 to 121.11.

CHAPTER II

Porcine animals

1. *Tuberculosis*

The single intradermal tuberculin test using avian tuberculin shall be carried out according to Annex B to 64/432/EEC, except that the site of injection shall be the loose skin at the base of the ear.

2. *Brucellosis*

The serum agglutination test and complement fixation test shall be carried out according to paragraphs A and B of Annex C to Directive 64/432/EEC.

3. *Aujeszky's disease*

The serum neutralization test shall be carried out according to the following protocol:

Serum:

All sera are heat-inactivated at 56 °C for 30 minutes before use.

Procedure:

The constant virus-varying serum neutralization test on microtitre plates employs Vero or other sensitive cell systems. Aujeszky's disease virus is used at 100 $TCID_{50}$ per 0,025 ml; inactivated undiluted serum samples are mixed with an equal volume (0,025 ml) of virus suspension. The virus/serum mixtures are incubated for two hours at 37 °C in the microtitre plates before the appropriate cells are added. Cells are used at a concentration which forms a complete monolayer after 24 hours.

Controls:

virus infectivity assay,
serum toxicity controls,
uninoculated cell culture controls,
reference antisera.

Interpretation:

The results of the neutralization test and the titre of the virus used in the test are recorded after three to seven days incubation at 37 °C. Serum titres less than 1/2 (undiluted semen) are considered negative.

4. *Transmissible gastroenteritis*

The serum neutralization test shall be carried out according to the following protocol:

Serum:

All sera are heat-inactivated at 56 °C for 30 minutes before use.

Procedure:

The constant virus-varying serum neutralization test on microtitre plates employs A72 (dog tumour) cells or other sensitive cell systems. TGE virus is used at 100 $TCID_{50}$ per 0,025 ml; inactivated undiluted serum samples are mixed with an equal volume (0,025 ml) of virus suspension. The virus/serum mixtures are incubated for 30 to 60 minutes at 37 °C in the microtitre plates before the appropriate cells are added. Cells are used at a concentration which forms a complete monolayer after 24 hours. Each cell receives 0,1 ml of cell suspension.

Controls:

virus infectivity assay,
serum toxicity controls,
uninoculated cell culture controls,
reference antisera.

Interpretation:

The results of the neutralization test and the titre of the virus used in the test are recorded after three to five days incubation at 37 °C. Serum titres less than 1/2 (final dilution) are considered negative. If undiluted serum samples are toxic to the tissue cultures, these sera may be diluted 1/2 before being used in the test. This will be equivalent to 1/4 final dilution of serum. Serum titres of less than 1/4 (final dilution) are considered negative in these cases.

5. *Swine influenza*

The haemagglutination-inhibition test shall be carried out according to the following protocol:

Procedure:

The tests are performed by standard methods (US Department of Health, Education and Welfare, *Immunology* series No 6)

To destroy non-specific inhibitors swine sera should be treated with either receptor-destroying enzyme (*Vibrio cholera* filitrate) overnight at 37 °C followed by heating at 56 °C for 30 minutes to destroy residual enzyme activity, or by treating with 25 % kaolin overnight at 4 °C (Clark and Casals, 1958, *American Journal of Tropical Medicine and Hygiene*, 7, 561).

After absorption with a 10 % suspension of chicken erythrocytes for one hour at 37°C, sera are tested against four haem-agglutinating units of the appropriate virus using 1 % chicken erythrocytes. Virus and serum are left in contact for 60 minutes at room temperature before the erythrocytes are added.

Interpretation:

Titres of 1/10 or greater are considered positive.

6. *Foot-and-mouth disease*

Tests for foot-and-mouth disease in porcine animals shall be carried out according to the protocols laid down in Chapter I, point 10.

7. *Swine vesicular disease*

The serum neutralization test shall be carried out according to the following protocol:

Serum:

All sera are heat-inactivated at 56 °C for 30 minutes before use.

Procedure:

The constant virus-varying serum neutralization test on microtitre plates employs IB-RS-2 cells or other sensitive cell systems. The G 27/72 or any other reference strain of virus is used at 100 $TCID_{50}$ per 0,025 ml. Sera for the test are diluted 1/4 and inactivated and a twofold dilution series prepared. The serum samples are mixed with an equal volume of virus suspension and incubated for one hour at 37 °C in the microtitre plates before adding 0,025 ml cell suspension containing 3×10^6 cells/ml.

Controls

virus infectivity assay,
serum toxicity controls,
uninoculated cell culture controls,
reference antisera.

Interpretation:

The results of the neutralization test and the titre of the virus used in the test are recorded after three to five days incubation at 37 °C. Serum titres are considered negative if there is no neutralization at a dilution of 1/11.

8. *Classical swine fever*

Tests for classical swine fever shall be carried out according to Annex I to Council Directive 80/217/EEC [1].

9. *Leptospirosis*

Tests for leptospirosis in porcine animals shall be carried out according to Chapter I, point 6 of this Annex.

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[1] OJ No L 47, 21. 2. 1980, p. 11.

ANNEX II

MINIMUM CONDITIONS FOR THE APPROVAL OF MARKETS FOR TRADE IN ANIMALS OF THE BOVINE OR PORCINE SPECIES INTENDED FOR EXPORT TO THE EUROPEAN COMMUNITY

1. Markets shall be supervised by an official veterinarian.
2. Markets shall each be situated at the centre of an area 20 km in diameter in which, according to official findings, for at least 30 days prior to their use as approved markets there has been no case of food-and-mouth disease and, where the markets are approved for porcine animals, no case of swine fever, swine vesicular disease or porcine enteroviral encephalomyelitis (Teschen disease).
3. Markets shall, before being used as approved markets, be cleansed and disinfected with a disinfectant officially authorized in the exporting country as effective in the control of the diseases mentioned in condition 2 above.
4. Assembly points, places of loading or other places through which bovine or porcine animals for export to the European Community may pass shall satisfy conditions 1, 2 and 3 of this Annex.
5. All bovine or porcine animals passing through approved markets shall fulfil the health conditions established for the importation of the relevant category of animal into the European Community.
6. Animals to be exported to the European Community which pass through approved markets must, within six days thereafter, be loaded and dispatched directly to the frontier of the exporting country:
   (a) without coming into contact with cloven-hoofed animals other than animals of the bovine or porcine species which fulfil the health conditions established for the importation of the relevant category of animal into the European Community;
   (b) segregated into consignments so that no consignment contains both animals for breeding or production and animals for immediate slaughter;
   (c) in transport vehicles or containers which have first been cleansed and disinfected with a disinfectant officially authorized in the exporting country as effective in the control of the diseases mentioned in condition 2 above and which are so constructed that faeces, urine, litter or fodder cannot flow or fall out of the vehicle during transportation.
7. Where the conditions for the export of animals to the Community require that a test be carried out within a specified period before loading, that period includes any period of assembly, up to six days, subsequent to the passage of the animals through approved markets.
8. The exporting country shall designate those markets which are approved for animals for breeding and production and those markets which are approved for animals for slaughter and shall notify the Commission and the competent central authorities of the Member States of the names and addresses of such markets.
9. The exporting country shall determine the procedure for official supervision of markets, assembly points and places of loading and shall ensure that such supervision is carried out.
10. The exporting country shall ensure that details of markets and assembly points are included in the relevant section of the health certificate which accompanies animals exported to the European Community.