EU DIAGNOSTIC MANUAL FOR
CLASSICAL SWINE FEVER (CSF) DIAGNOSIS:
TECHNICAL PART
(THIRD DRAFT JUNE 2007)

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Purpose
The purpose of the technical manual in hand is to support and harmonize CSF diagnosis. The manual shall be understood as guideline. In general, all methods and protocols have to be validated in the respective laboratory and the guidelines according to ISO/IEC 17025 must be fulfilled. Apart from the methods mentioned, any fully validated test protocol might be used.

This version of the diagnostic manual aims at tests performed on non vaccinated pigs. It has to be stressed that (marker) vaccinated animals may or will give a positive reaction in the majority of the below mentioned serological tests.

Suitable cell lines

Porcine kidney cell line PK15
The PK 15 cell line is suitable for all test systems for CSF diagnosis e.g. virus isolation, virus replication, and virus neutralisation tests.

The cell line can be obtained from the CRL. It was established there by single cell cloning. The cells are supplied free of mycoplasma, and pestiviruses; a PCV-1 contamination is known for this cell line.

Cell culture medium
For the preparation of stock cells, Eagle’s Minimum Essential Medium (EMEM) with 5% foetal calf serum (FCS) is routinely used. In contrast, for all virological and serological tests from diagnostic material, virus multiplication using virus containing cell culture supernatants, and for cultivation of virus in a 24 or 96 well culture dish, EMEM with 10% FCS is the ideal medium. Antibiotics and antimycotics should be added (Penicillin/Streptomycin, Gentamycine, Fungizone or others) to the medium used with diagnostic samples which might be contaminated with bacteria or fungi. This might also be useful in any open culture system such as macro or micro titre plates.

The FCS must be carefully checked for BVDV by virus isolation and for BVD antibodies by virus neutralisation test before use. Although it does not influence the outcome of serological tests, acceptance of viral genome is still under discussion among the laboratories.

It is not sufficient to rely on the manufactures certification only.
Cultivation of PK15 cells
For multiplication, PK15 cells are kept in 75 cm² (250 ml) plastic culture flasks with app. 20 ml cell culture medium. Two passages weekly are ideal, but one passage weekly is sufficient. The routine splitting ratio is 1:5.
There are different protocols in use to trypsinise and seed the cells. The most widely used protocols are listed below:

Cultivation without centrifugation:
- Remove medium from a 250 ml culture flask
- Wash monolayer (once or) twice with 5 ml of adjusted trypsin/versen (ATV) solution
- Remove ATV and replace with 2 ml fresh ATV
- Incubate at 37°C for 15 min or until cells are detached
- Fill up with 8 ml of culture medium containing 5% FCS to get a total volume of 10 ml.

Cells are seeded in the different dishes as described below.

Traditional protocol:
- Remove medium and wash with PBS/Versene.
- Incubate 10 min at 37°C with PBS/V.
- Add 0.1 ml of 1% Trypsin-solution.
- Incubate 10-20 min at 37°C until cells are removed from the dish.
- Add medium with FCS to stop trypsin reaction.
- Centrifuge cell suspension at 194 x g for 10 min at room temperature and discard supernatant.
- Resuspend pellet in 10 ml or other appropriate medium volume.

Seeding of different culture dishes
A confluent monolayer of a 75 cm² culture flask contains an average of 8.5 x 10⁶ cells.
If the cell pellet of one flask, obtained by trypsination is resuspended with 10 ml medium, the following amounts are seeded in the different cell culture vessels:
<table>
<thead>
<tr>
<th>Dish</th>
<th>10 ml cell suspension</th>
<th>ml medium per vessel</th>
<th>Total cells / vessel (average)</th>
<th>Cells / ml (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 cm² flask</td>
<td>2 ml</td>
<td>20-25</td>
<td>1.6 x 10⁶</td>
<td>8.5 x 10⁴</td>
</tr>
<tr>
<td>25 cm² flask</td>
<td>0.5 ml</td>
<td>5-7</td>
<td>4.2 x 10⁵</td>
<td>8.5 x 10⁴</td>
</tr>
<tr>
<td>Culture tube</td>
<td>0.1 ml</td>
<td>1</td>
<td>8.5 x 10⁸</td>
<td>8.5 x 10⁴</td>
</tr>
<tr>
<td>24 well plate</td>
<td>2 ml / dish</td>
<td>24</td>
<td>1.6 x 10⁶</td>
<td>8.5 x 10⁴</td>
</tr>
<tr>
<td>96 well plate</td>
<td>2 ml / dish</td>
<td>5-7</td>
<td>1.6 x 10⁶</td>
<td>3 x 10⁵</td>
</tr>
</tbody>
</table>

Regular screening for mycoplasmas should be performed. The frequency has to be determined individually. In case of increased doubtful results or reduced cell growth, the cell culture should be examined for mycoplasmas.

**Sheep foetal thymus (SFT-R) cell line**

For isolation and cultivation of BDV, a sheep fetal thymoid cell line (SFT-R/CCLV Rie043) is available. The main use is the differential virus neutralisation test against BDV. The cell line is available at the cell collection (CCLV) of the Friedrich-Loeffler-Institute, Island of Riems, Germany.

**Cell culture medium**

For the preparation of all culture dishes and replication of the stock cells EMEM with the modifications according to Dulbecco and Freeman (EDulb) with 5 % FCS is routinely used. Antibiotics and antimycotics should be added (Penicillin/Streptomycin, Fungizone or others) to the medium used with diagnostic samples which might be contaminated with bacterial or fungi. The FCS is carefully checked for BVDV by virus isolation and for BVD antibodies by virus neutralisation test before use. It is not sufficient to rely on the manufactures certification only.

**Cultivation of SFT-R cells**

For multiplication, SFT-R cells are kept in 75 cm² (250 ml) plastic culture flasks with 20 ml cell culture medium. One passage weekly is sufficient; two passages weekly are possible if necessary. The routine splitting ratio is 1 : 3.

- Remove medium from culture flask
- Wash monolayer with 5 ml of ATV solution for 30 seconds.
- Remove ATV and replace with 2 ml fresh ATV
• Incubate at 37°C for 15 min or until cells are detached
• Fill up with 7 ml of culture medium containing 5 % FCS to get a total volume of 9 ml.
• Routine splitting ratio 1 : 3
• Seed cells with a density of 1.5 x 10^5 / ml in a 96 well dish
• Seed cells with a density of 9 x 10^4 / ml in cell culture tubes or 24 well dishes

Regular screening for mycoplasmas should be performed. The frequency has to be fixed individually. In case of increased doubtful results or reduced cell growth, the cell culture should be examined for mycoplasmas.

**Other cell lines**

Several other porcine cell lines are in use for CSF diagnosis. Their suitability for CSF diagnosis is under investigation. The permanent porcine kidney cell lines SK-6 and Rie 5-1 have proven to be suitable for CSF diagnosis. A Swine Testis Endothelial (STE) cell line does grow with horse serum and is therefore less vulnerable to BVD contamination by fetal calf serum. Both, STE and Rie 5-1 cells were shown to propagate some virus strains that do not grow well on PK15 cells.

For BVDV cultivation, several susceptible bovine cell lines are suitable e.g. Madin Darby bovine kidney (MDBK), embryonic bovine trachea (EBTr) and bovine turbinate cells. Primary cells (porcine or bovine) in general have the disadvantage of being less homogenous within one batch and between batches of different laboratories. Nevertheless the sensitivity of these cells might be higher than of permanent cells.
**Reagents**

**PBSV:**
- NaCl     8.0 g/l
- KCl     0.2 g/l
- Na2HPO4 x 12 H2O   2.37 g/l
- KH2PO4    0.2 g/l
- Versene  0.2 g/l

**ATV:**
- NaCl     8.0 g/l
- KCl     0.4 g/l
- Dextrose 1.0 g/l
- Na HCO3   0.58 g/l
- Versene  0.2 g/l
- Trypsin  0.5 g/l

Antibiotics if considered necessary.

**CSF virus isolation**

**Principle of test**
Organ or leucocytes preparations are incubated on CSFV susceptible cells to allow the attachment and replication of the virus. Since growth of the virus does not cause a cytopathic effect, its presence must be demonstrated by an immunostaining method. Cells are fixed and the viral antigen is detected with a peroxidase or fluorescein labelled CSFV-specific antibody. Immunostaining may be carried out after one or two virus passages.

**Cell culture systems for CSFV isolation**
Cell cultures of PK15, SK-6 or Rie 5-1 cells which are 50 – 80 % confluent (1-3 days old) should be used for inoculation with test samples.
Organ preparations

Suitable organ samples are described in the Diagnostic Manual, Chapters V (B) and VI (A and B), e.g. tonsil, spleen, kidney, lymph nodes or ileum. Samples should be kept chilled and processed as soon as possible. Traditional protocols using a mortar and homogenising machines might be used to process the samples. Commonly used methods are listed below.

Traditional protocol

Organ samples of 1 cm³ are homogenized in a mortar in 9 ml cell culture medium containing antibiotic solution to produce a 10 % organ preparation. Smaller sample sizes can be homogenized with less medium in order not to dilute the virus too much but to produce a 10 % organ preparation. Sterilised sand can be added to facilitate homogenisation.

- The preparation is left at room temperature for one hour.
- Centrifugation for 15 min at 2500 g.
- The supernatant is used for inoculation of cell cultures.

A 1:10 and a 1:100 dilution can be processed in parallel, in case of cytotoxic effects. Sterilfiltration can be performed, if considered necessary using syringe filters (0.45 µm followed by 0.22 µm).

Alternative protocols (exemplary)

Homogenising machines utilizing for example ceramic beads (e.g. Ribolyser®, Fastprep®) can be used to mince organ material. The advantage is that the tissue is homogenised in a closed system, but high speeds will heat the sample and thus possibly affect the virus.

- Special tubes are filled with 1 ml of the appropriate buffer or cell culture medium (preferable Earl’s with lactalbumine (ELA) medium)
- Organ samples are cut to pieces with a scalpel and a pea sized piece is put into each tube
- Tubes are closed and put into the homogenising machine
- The appropriate speed is 4 m/45 s
- After the first run, the homogenising process is macroscopically assessed
- If required, the process can be repeated
- Finally, the material of these tubes is pooled and processed as described above (1 h at room temperature followed by centrifugation)

Another alternative is to cut the material into fine pieces and put it in a Stomacher®.
Leukocyte preparation

Leukocyte preparations can be obtained from EDTA and heparin anticoagulated blood.

EDTA anticoagulated blood

- 0.5 ml of Dextran solution are added to 10 ml EDTA blood and left at room temperature for one to three hours until the leukocytes are visibly separated. Smaller sample volumes should be processed in the same way with 0.5 ml Dextran.
- The supernatant containing the leukocytes is collected and centrifuged 10 min at 437 g.
- The pellet is resuspended in 3-5 ml PBSM, centrifuged and washed again in the same way.
- Finally the pellet is resuspended in 2 ml PBSM for further use or storage.
- In case the leukocyte suspension is inoculated simultaneously with susceptible cells, it should be frozen at –20°C shortly to lyse the leukocytes and avoid cytotoxic effects.

Other (commercial) preparations may be used, if applicable according to the manufacturers instruction, e.g. Ficoll-Paque.

Heparin anticoagulated blood

- Erythrocytes are lysed by adding 20 ml of NH4Cl solution (0.84%) to 10 ml of Heparin blood sample (2:1) and left at room temperature for 15-30 min. In smaller sample sizes the volume of NH4Cl is adapted accordingly (2:1).
- The solution is centrifuged at 1000 g.
- The pellet is resuspended in 5 ml PBSM, centrifuged and washed again in the same way.
- Finally the pellet is resuspended in 2 ml PBSM for further use or storage.
- In case the leukocyte suspension is inoculated simultaneously with susceptible cells it should be frozen at –20°C shortly to lyse the leukocytes and avoid cytotoxic effects.

Whole blood samples:

10 ml of EDTA or heparin anticoagulated blood are subjected to a freeze and thaw cycle in order to lyse the cells. After thawing, the lysate is inoculated directly on the cell culture as described below. Plasma from citrated blood and heparinised whole blood also works fine.
Test procedure

Screening

• 200-300 µl of the organ, leukocyte preparation or blood lysate are inoculated on a 50-80 % confluent cell culture in multi dish plates or Leighton tubes with cover slips, enough to cover the monolayer. Duplicate cultures of each sample should always be prepared.

• The cell cultures are incubated at 37°C for 1 - 2 hours to allow viral attachment.

• The cell cultures are washed once with PBSM and overlaid with fresh medium. Alternatively the cell culture dish can be filled up directly, if a cytotoxic effect is unlikely.

• The cell cultures are incubated up to 72 (96) hours at 37°C in a CO2 incubator.

• Simultaneous inoculation is possible, if the sample is fresh and a cytotoxic effect is unlikely.

• Positive and negative controls must also be processed in the same way. As a positive control, CSFV reference strains might be used.

• Cells are fixed and stained as described in the Chapter ‘Immune Labelling’.

Virus isolation over two passages

• 200-300 µl of the organ, leukocyte preparation or blood lysate are inoculated on a cell culture tube. Always duplicate cultures of each sample should be prepared.

• The cell cultures are incubated at 37°C for 1 - 2 hours.

• The cells are washed twice with PBSM and are further cultivated for 72 hours at 37°C. EMEM with 10 % FCS is the ideal medium for virus growth.

• Simultaneous inoculation is possible, if the sample is fresh and a cytotoxic effect is unlikely.

• The cell culture tubes are frozen at -80°C for at least one hour (freeze and thaw cycle).

• The culture tubes are then thawed and centrifuged 10 min at 778 g.

• 200-300 µl of the supernatant are incubated for 1-2 hours on a well of a multi dish plate or Leighton tube as described above.

• After refilling the culture medium, the plates are incubated for another 72 to 96 hours.

• Positive and negative controls must also be processed in the same way.

• Cells are fixed and stained as described in the Chapter Immune Labelling.
In case a slow growing isolate is suspected, a second passage in a culture tube can be done, leading to a third passage in a culture dish.

**Material**

**Dextran solution 5%:**
Dextransulfat-Na, MW 500.000 5 g
1.5 % EDTA-Solution ad 100 ml
Dissolve at 45°C and autoclave before use.

**PBSM**
NaCl 8.0 g
KCl 0.2 g
Na2HPO4 2.37 g
Dextrose 1.0 g
Aqua bidest. ad 1000 ml

**Antibiotic solutions**

*Penicilline/Streptomycin*
Na-Benzylpenicilline $10^7$ I.U.
Streptomycinsulfate 10 g
PBS ad 100 ml
This solution is used 1 ml in 1 l medium for routine use or 10 ml in 1 l medium for incubation of organ samples.

*Glutamine based antibiotic cocktail*
Streptomycinsulfate 11.25 g dissolved in 390 ml Aqua dest.
Na-Benzylpenicilline $9 \times 10^6$ I.U. dissolved in 90 ml Aqua dest.
L-Glutamine 26.28 g dissolved in 420 ml Aqua dest.
Mix and filter the three solutions.
Fungizone $9 \times 10^5$ I.U. dissolved in 100 ml Aqua dest.
Add to the above filtrate and store at ~20°C in 10 ml aliquotes until use.
Application: 10 ml in 1 l medium
Immune Labelling for the detection of CSF virus in cell cultures

In general, CSF does not induce a cytopathic effect. Consequently the CSFV infected cells have to be immunologically labelled in order to detect the virus antigen in the cytoplasma of the cells. Direct and indirect staining methods as well as mono- and polyclonal antibodies are available. The working dilution of the conjugates should combine a maximum of signal with a minimum of background staining and has to be evaluated in each laboratory.

Cell Fixation

The choice of the fixative will depend on whether the cultures are grown on glass or plastic surfaces. For cultures grown on glass surfaces, fixation in 100 % acetone for 5 min is appropriate. In case plastic surfaces are used different fixation methods are available:

Heat fixation

- Remove culture medium very thoroughly
- Wash once with 1/3 of PBSM and
- Incubate the plates at 70-80 °C for 2-3 hours (SFTR-cells may require app. 5 hours)

Alternative protocols

- Add 100 µl/well of acetone/methanol (1:1) solution and leave for 10 min at room temperature or
- Fix in 20 % acetone for 10 min. Drain plates thoroughly, and then dry under a bench lamp for 4 hours at 25-30°C
- Alternatively, wells can be washed once in ice-cold 99.9% ethanol and left with 99.9% ethanol for 45 min at 4°C. Staining should be done immediately.

Fixed plates may be stored before staining for several days in dry environment and longer periods at – 20°C in a sealed bag.

Other fixation procedures bear the risk of interfering with the penetration of the monoclonal antibody.
Immune labelling with peroxidase staining

Direct labelling

- Rinse the plates once in PBS-Tween.
- Add to each well the working dilution of a pestivirus conjugate in PBS Tween.
- In a 24 well dish 200 µl/well and in a 96 well dish 50 µl/well of conjugate are suitable amounts.
- Incubate for 1 hour at 37 °C (without CO₂) in a moist chamber.
- Wash the plates three times with PBS-Tween and once with Aqua dest.
- Add to each well 200 or 50 µl of chromogen-substrate solution and stain for 15-30 minutes at room temperature.
- Discard the chromogen-substrate solution and wash with 1/3 PBS/H₂O.
- Fill the wells with Aqua dest. and read the test by low-power microscopy.
- **The cytoplasm of infected cells is stained dark red.**

Indirect labelling

- Add to each well the working dilution of a pestivirus specific antiserum or suitable monoclonal hybridoma supernatant
- Incubate for 1 hour at 37 °C (without CO₂) in a humid chamber.
- In a 24 well dish 200 µl and in a 96 well dish 50 µl of conjugate are suitable amounts.
- Wash the plates three times with PBS-Tween and once with Aqua dest.
- Add to each well the working dilution of a commercial antispecies peroxidase conjugate in PBS Tween and incubate for 1 hour at 37 °C (without CO₂).
- Wash the plates three times with PBS-Tween and once with Aqua dest.
- Perform incubation with second antibody, if required by manufacturer.
- Add to each well 200 or 50µl of chromogen-substrate solution and stain for 15-30 minutes at room temperature.
- Discard the chromogen-substrate solution and wash with 1/3 PBS/H₂O.
- Fill the wells with Aqua dest. and read the test by low-power microscopy.
- **The cytoplasm of infected cells is stained dark red.**

Incubation times may vary according to differences between conjugates. Horse serum or other blocking reagents might be used to block the reaction and reduce background staining. In this case, e.g. 4 % horse serum is added to the second antibody solution.
Immune labelling with FITC (Fluorescein) staining

Immune labelling with FITC staining can be done on cover slips, plates or chamber slides as described below:

- Rinse the cover slips, plates or chamber slides once with washing buffer.
- If necessary, the working dilution of the FITC conjugated antibody in washing buffer can be filtrated using a millipore filter to remove FITC crystals.
- Overlay the fixed cells with FITC conjugate in a suitable dilution (in washing buffer)
- Incubate for 30-60 min at 37 °C in a moist chamber. The cells should be completely covered with conjugate. Some conjugates are not compatible with CO₂ incubators.
- Rinse in washing buffer 3 times for 5 minutes.
- Rinse once in Aqua bidest.
- Place a drop of mounting buffer onto the cells. If necessary, use a cover slip to cover the cells
- Investigate for cytoplasm fluorescence by UV microscopy.

In case labelling is done indirectly, one incubation step with a FITC labelled antispecies antibody has to be included.

To avoid non-specific binding 0.1% CSF antibody negative pig serum can be added to conjugate dilutions.
Material

For peroxidase staining

*PBS-Tween:*

1 l PBS containing ~0.01% Tween20 (2-3 drops)

*1/3 PBS:*

PBS : H2O = 1 : 3

*Antispecies antibodies:*

Commercially available, e.g. rabbit anti-mouse peroxidase, RAMPO

*Sodium Acetate-Buffer (pH 5.0; 0.05 M):*

Sodium Acetate-Trihydrate 6.804 g
Aqua bidest. 1000 ml

The pH can be adjusted using acetic acid.

*Chromogen-substrate solution:*

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Amino-9-Ethylcarbazol (AEC)</td>
<td>20 mg</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>3 ml</td>
</tr>
<tr>
<td>Sodium Acetate-Buffer</td>
<td>ad 50 ml</td>
</tr>
<tr>
<td>H2O2 (3 %)</td>
<td>0.4 ml</td>
</tr>
</tbody>
</table>

A stock solution can be prepared using AEC and Dimethylformamide.

For FITC staining

*Washing buffer:*

PBS (pH 7.4 - 7.6, physiological saline buffered with 0.01 M phosphate)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.78 g/l</td>
</tr>
<tr>
<td>Na2HPO4 x 12H2O</td>
<td>3.58 g/l</td>
</tr>
</tbody>
</table>

(pH adjusted with 1M KH2HPO4)

*Mounting buffer*

Washing buffer with 20% glycerine (buffered glycerine) or commercial non-fading mountant.
**Immune labelling using peroxidase**

1. Rinse wells once with 1/3 PBS, add 50 or 200 µl/well of conjugate and incubate for 1 h at 37 °C

2. Wash plate 3 x with PBS-Tween and 1 x with Aqua bidest., add 50 or 200 µl/well chromogen-substrate, incubate 15-30 minutes at RT

3. Discard chromogen-substrate, wash and add Aqua bidest., read by low power microscopy
Differential Diagnosis of virus isolates

In the case where a pestivirus is isolated during virus isolation, it has to be characterised. Characterisation can be achieved using CSFV specific RT-PCR preferably in combination with subsequent genetic typing. Another traditional method uses monoclonal antibodies specific for CSFV, BDV and BVDV. CSFV specificity is crucial.

List of available antibodies

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Pestivirus</th>
<th>CSF</th>
<th>BVD</th>
<th>BD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL, Hannover</td>
<td>Yes, mAB C16</td>
<td>Yes, mAB HC34</td>
<td>Yes, mAB CA3, CA34</td>
<td>No</td>
</tr>
<tr>
<td>IDEXX Laboratories, CH</td>
<td>Yes, PO-conj.</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cedi Diagnostics, NL</td>
<td>Yes, PO-conj.</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>VLA, UK</td>
<td>Yes, W54 Pool</td>
<td>Yes, WH303</td>
<td>Yes, WB</td>
<td>Yes, WS</td>
</tr>
</tbody>
</table>

The CRL can supply the monoclonal antibodies (mAB) in small quantities in form of hybridoma supernatants. At the moment, these materials can only be supplied for research purposes as they are not licensed for diagnostic purposes. Commercial availabilities change frequently. Please observe the market. This list is not a recommendation of certain products.

References


Fluorescent antibody test on tissue sections

Principle of test

Classical swine fever virus (CSFV) antigen is demonstrated in thin cryostat sections of organ material. The cryostat sections are mounted on a microscope slide and stained directly with anti-CSF immunoglobulin conjugated to fluorescein (FITC-conjugate). The sections are examined for fluorescence by UV microscopy.

Suitable organ samples are described in the Diagnostic Manual, Chapters V (B) and VI (A, 1.) e.g. tonsil, spleen, kidney, ileum or lymphnodes.

The quality of the test mainly depends on experienced personnel and well-defined test conditions.

Test procedure of direct FAT

- Cut out a piece of tissue of app. 1 x 1 x 0.5 cm and mount it with a cryo-embedding compound or distilled water on a cryostat mounting block (chuck). Tonsils have to be embedded to allow a crosssection.
- Freeze the piece of organ onto the cryostat chuck. The freezing temperature of the cryostat should be −15 to −20°C.
- Ideal is shock-freezing of the tissue in n-Heptan cooled with liquid N₂.
- Cut sections of maximum 5 µm thickness and mount them onto microscope slides which have been previously cleaned with alcohol.
• Prepare several slides with sections from the same tissue.
• Dry the mounted sections at room temperature for 20 minutes.
• Fix the mounted sections for 10 minutes in acetone (analytic grade) at –20°C.
• Immerse the section briefly in washing buffer, remove excess fluid with tissue paper and place them on a frame in a humid incubation chamber.
• Remove a fixed control positive and control negative section from the deep freeze (-70 °C) and process in parallel.
• Dispense the FITC-conjugate at the suitable working dilution in washing buffer onto the entire section
• Close the moist chamber and incubate in the dark for 30 minutes at 37 °C.
• Check, that the conjugate solutions have not evaporated and dried the tissue.
• Wash the sections three times for 10 minutes at room temperature with washing buffer
• Immerse the section briefly in distilled water.
• If necessary, counterstain in Evans Blue for 30 seconds.
• Carefully remove excess fluid with tissue paper and place a cover slip with mounting buffer onto the section.
• Remove excess mounting fluid with tissue paper and examine the sections for fluorescence by UV microscopy.

**Controls**

Negative and positive control sections must be included in each series of organ samples to be examined. The control sections can be prepared in advance and stored after acetone fixation for 2-3 years at -70 °C.

**Interpretation**

Any sample showing specific cytoplasmic reaction (brilliant green fluorescence) shall be considered positive for pestivirus.

A negative FAT result does not necessarily rule out CSF in all cases. In some cases during the terminal stage of the disease a positive reaction can be masked by neutralizing antibodies which are already induced by the organism. In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence.
Pigs vaccinated with modified live virus strains may yield a positive FAT result for two weeks after vaccination. Strains of modified live virus vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Depending on the type of vaccine, it may be possible to carry out differential staining with vaccine-specific monoclonal antibodies. Non-specific staining does also occur, which could lead to false positive results. The experience of the reader is crucial for the interpretation of test results.

Pigs infected with Bovine viral diarrhoea (BVD) or Border disease (BD) viruses can give false positive results when a FITC-conjugate prepared from a polyclonal antibody specific for pestiviruses has been used. For this reason, it is recommended that, especially in CSFV-free areas, duplicate samples should be examined from FAT positive cases using monoclonal antibodies which can distinguish CSF virus from BVD or BD viruses. Alternatively, confirmatory diagnosis should await virus isolation in cell culture with subsequent typing by monoclonal antibodies or CSF specific PCR.

**FITC-conjugate**

The quality of the FITC-conjugate determines the quality of the reaction. It is recommended that primary diagnosis is carried out with FITC-conjugates prepared from a polyclonal antibody to CSF. This will not distinguish between the antigens of different pestiviruses, but does provide assurance that minor variant viruses will not be missed. FITC-conjugates should be prepared from hyperimmune serum prepared in specific pathogen free pigs. The serum should be free from any antibody which could affect the specificity or quality of the specific CSF reaction. In case of a positive result, virus isolation should be carried out or staining should be repeated with a CSF specific antibody.

Different commercial FITC-conjugates are available. Commercial availabilities change frequently. Please observe the market.

The working dilution of the conjugate should combine a maximum of signal with a minimum of background staining and has to be evaluated in every laboratory.

Commercial availabilities are frequently changing. Please observe the market. This list is not a recommendation of certain products.

**References**

**Material**

*Washing buffer:*

PBS (pH 7.4 - 7.6, physiological saline buffered with 0.01 M phosphate)

- NaCl 8.78 g/l
- Na2HPO4 x 12H2O 3.58 g/l
  
  (pH adjusted with 1M KH2HPO4)

*Evans Blue*

- Stock solution 1:100 in Aqua bidest.
- For use dilute 1:2000 in PBS

*Mounting buffer*

Washing buffer with 20% glycerine (buffered glycerine) or a commercial non-fading mountant.
Demonstration of viral antigen in cryostat sections

(Direct Fluorescent Antibody Test - FAT)

1. Freeze tissue (1 x 1 x 0.5 cm) onto cryostat table using one drop of water.
2. Cut sections of 5 µm, 2-3 per tissue.
3. Mount section onto slide.
4. Air dry (20 min).
5. Fix in acetone at -20°C for 10 min.
6. Rinse in buffer.
7. Stain for 30 min. at 37°C with FITC conjugate in moist chamber.
8. Wash 3 x 10 min. with buffer.
9. Rinse in distilled water.
10. Place coverslip with mounting buffer onto section.
11. Examine by fluorescence microscopy.
CSF antigen ELISA

Commercially available antigen capture ELISAs can be used to analyse blood, organ, plasma, or serum samples. Most of them detect CSF viral glycoprotein E\textsuperscript{rev}. Although this is an easy to handle, rapid method that yields a result after about four hours and can be fully automated, its performance is hampered by a rather low sensitivity and specificity. Therefore, the antigen capture ELISAs should never be used to test individual animals and positive results should be confirmed with another antigen detection test (i.e. virus isolation or RT-PCR). Each NSFL is in charge for the licensing of different batches of commercial CSF antigen ELISAs. The CRL does not recommend a certain antigen ELISA. At present, CSF antigen ELISAs can be obtained from:

- Synbiotics, F
- CEDI Diagnostics, NL
- IDEXX Laboratories, USA and CH

Diagnostic RT-PCR

Introduction

Procedures are described for the detection of CSFV genome in anticoagulated whole blood, serum, tissues or cell cultures.

Since the introduction of PCR and the availability of adequate laboratory equipment, this technology has become an essential tool in research and diagnostic laboratories. The first protocols for the detection of nucleic acid from CSFV using RT-PCR were published in the early nineties (Liu 1991, Katz 1993, Roehe 1991). In the meantime, it has become a standard technology in most laboratories and is used in routine diagnosis of CSFV. RT-PCR has been found to be the most sensitive method for detection of CSFV (Dewulf 2004, Handel 2004). In comparison to virus isolation in cell culture, the viral nucleic acid can be detected earlier after infection and for a longer period in cases where the pigs recover. RT-PCR protocols that are used as a diagnostic tool must prove to be at least as sensitive as virus isolation. Pools of samples can be tested but sensitivity should be as high as the sensitivity of virus isolation from single samples.
RNA-Extraction

RNA isolation is one of the most often underestimated critical steps when RT-PCR is used for mass sample screening. Two points in the isolation process have to be carefully considered: the treatment and handling of the samples prior to RNA isolation and the storage of the isolated RNA (Blacksell 2004). Since the first step in the procedure is lysis of the cells by adding a strong denaturant (such as GITC, LiCl, SDS, phenol) that inactivates the enzymes including the RNases, it is typically prior to and after the isolation, when RNA integrity is at risk. Pretreatment of the samples and buffers for optimal performance of the commercially available kits are optimised for the kind of samples used (cell culture supernatant, serum, tissue, blood), and efficient RNA isolation can only be achieved if the right kits are used for the right samples.

Two technologies are widely used for nucleic acid isolation for RT-PCR: the liquid based and the silica or glass based methods. For both options, commercial kits are available and should be carried out as described by the manufacturer.

The liquid-based methods are enhancements of the original nucleic acid isolation using guanidinium thiocyanate/acid phenol:chloroform (Chirgwin 1979, Chomczynski 1987). Ready-to-use reagents are commercially available under different brand names (TRIZOL®, TRI Reagent®, Stratagene RNA Isolation Kit®, etc.), allowing single-step disruption/separation procedures.

Silica- or glass-based matrices or filters selectively absorb nucleic acids in the presence of chaotropic salts (Boom 1990), which immediately inactivate the RNAses. After washing off the remaining components of the lysate using a high-salt buffer, bound RNA is eluted with water. In many commercially available kits, the glass or silicon filter or fleece is housed in spin columns or in 96-well plates. Solutions are driven through the filter by centrifugation or under vacuum. Some kits also include a proteinase K or a DNAse incubation for removal of proteins and DNA, respectively. Although the yield of RNA is lower than that obtained with guanidinium thiocyanate/acid phenol:chloroform extraction (Scheibner 2000), the silica-based kits can be adapted to be used in nucleic acid extraction robots, thus allowing full automation of CSFV diagnosis. In addition, no hazardous reagents are needed.
As an example, at present suitable kits are:

- Cells grown in a monolayer: RNeasy Mini Kit (Quiagen)
- EDTA and Heparin whole Blood: QIAamp RNA blood mini kit (Quiagen)
- Serum: High Pure viral RNA (Roche)
- Cell culture supernatant: QIAamp viral RNA kit (Qiagen)

Extracted RNA should be held on ice until used in RT-PCR or stored at -70°C.

**Reverse transcription - polymerase chain reaction (RT-PCR)**

After extraction from the diagnostic sample the RNA has to be transcribed to cDNA that can then be amplified by PCR (RT-PCR). Evaluation of PCR or RT-PCR can be performed either by agarose gel electrophoresis (standard PCR), or by real-time techniques (qPCR or RT-qPCR).

**Gel-based PCR protocols**

After RT-PCR, the reaction mixture containing the amplicons is subjected to electrophoresis in agarose gels. Double-stranded DNA is visualised under ultraviolet (UV) light after incubation with a fluorescent intercalating stain, generally with ethidium bromide. By including a DNA marker, the size of the amplicon can be estimated and eventual false amplicons and primer-dimers become visible.

A great number of standard RT-PCR protocols for diagnosis of CSF have been published and are in use in many laboratories and national reference centres. The most widespread protocol amplifies a 288 bp fragment of the 5’ non translated region (5’-NTR) and is pestivirus-specific (Primers 324/326) (Vilcek 1994). Widely in use are also the CSFV-specific RT-PCR protocols targeting the E2 glycoprotein gene (Katz 1993), a 174 bp fragment of the NS5B non-structural protein gene (Diaz-de-Arce et al., 1998), and 108 bp of the 5’-NTR of the CSFV genome (Aguero et al., 2004). This last RT-PCR is a multiplex-PCR with two sets of primers, the second set was designed for amplification of 257 bp of the African swine fever genome. The RT-PCR targeting the E2 glycoprotein is a nested PCR, amplifying fragments with 308 and 172 bp, respectively (Katz 1993). Two other CSFV-specific RT-PCR protocols were designed to amplify fragments of the genome to be sequenced for genetic typing of new virus isolates. One of them amplifies a 521 bp fragment covering a part of the 5’-NTR and of the Npro gene (Greiser-Wilke et al., 1998) and the second one 671 bp of the E2 glycoprotein (Paton 2000). As genetic typing has become indispensable for epidemiological tracing of the isolates causing new outbreaks (Moennig 2003, Frias-Lepoureau et al., 2002), gel-based RT-
PCRs will continue to be useful to check correct amplification before sequencing the products.

**Real-time PCR protocols**

Real-time PCR (qPCR) or RT-qPCR detects the accumulation of amplicon during the exponential phase of the reaction, where it is still linear. This provides a distinct advantage over gel-based PCR detection, where only the end-point of the PCR reaction is evaluated. Although there are many different fluorogenic mechanisms (Belak 2005, Mackay 2002, Reynisson et al., 2005), in practice only two of them are currently used for CSFV diagnosis.

1. **Direct labelling of the RT-qPCR products using SYBR green**

Providing that the RT-PCR is optimised and no non-specific amplicons or primer-dimers arise, it has been found that virtually any PCR can be evaluated in real-time by using a fluorescent intercalating dye, such as SYBR green (Bente et al., 2002; Ponchel et al., 2003). This dye does not bind to single-stranded DNA, but fluoresces very brightly when it is bound to double stranded DNA. At the end of the amplification, the melting point of the product can be determined. Thus, the specificity of the reaction can be monitored, as differing melting points indicate the presence of contaminating products which can be due to contamination, mispriming, primer-dimers, or some other artefacts. SYBR green in RT-qPCR for detection of the CSFV genome has been successfully applied with the Pestivirus-specific primers 324/326 (Vicek 1994) in several laboratories. Another RT-qPCR protocol was applied for quantification of CSFV in serum from experimentally infected pigs (Uttenthal 2003).

2. **Indirect monitoring of the RT-qPCR using TaqMan probes**

TaqMan probes used in the 5’ nuclease assays are oligonucleotides that contain a fluorescent dye usually on the 5’ base and a quenching dye on the 3’ base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule (FRET = Förster or fluorescence resonance energy transfer) (Chen 1997, Hiyoshi 1994). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5’ exonuclease activity cleaves the probe (Holland 1991). The reporter dye starts to emit fluorescence which increases in each cycle in proportion to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (Dorak
All TaqMan assays for detecting the CSFV genome published to date amplify fragments of the 5’-NTR (Hoffmann 2006, McGoldrick 1999, Risatti 2003, Risatti 2005). The commercial availability from companies such as Applied Biosystems, Biomol, Qiagen, Stratagene and many others of PCR master mix kits for one-tube or two-tube (separating reverse transcription and the PCR) reactions that can be used for standard PCR and for the different chemistries in qPCR have greatly facilitated application of this method for handling large quantities of samples. Besides the nucleic acid, only the primers and probes and the polymerase which is supplied by the manufacturer have to be added, thus reducing the probability of contamination and of pipetting errors.

To date, only three of the TaqMan assays include a control for the RNA isolation and reverse transcription, one of them by integrating an exogenous control (Hoffmann 2006). The other two assays are kits developed by Laboratoire Service International (LSI, Li Bois Dieu, 1 Bis Alée de la Combe, 69380 Lissieu, France) (Taqvet CSF) and ADIAGENE (38 Rue de Paris, 22000 Saint Brieuc, France) (Adiavet CSF) which obtained an agreement to be commercialised in France, after they had been validated by the French CSF National Reference Laboratory (Le Dimna 2006). They also amplify the 5’ NTR and use the cellular housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

**Quality control**

RT-PCR is only diagnostically reliable when carried out in a suitably equipped and organized laboratory operated by properly trained and supervised staff. False positive results due to contamination can be fatal. It is essential to achieve complete separation of so-called ‘clean’ and ‘dirty’ procedures.

- Preparation of reagents and master mixes must be carried out in a facility that has no direct or indirect contact with any materials potentially contaminated with pestivirus RNA or DNA.
- A separate facility is needed to prepare samples and extract RNA. This must be set up to avoid both extraneous contamination and cross-contamination between test samples.
- An entirely separate facility is needed for analysis of PCR products.
- Strict codes must be enforced to ensure that personnel do not accidentally carry-over materials from one facility to another.
• Inclusion of negative and positive controls in all tests.
• The controls are processed in exactly the same way as the real samples.

Negative controls
• As a minimum, one negative control for each batch of samples tested together.

Positive controls
• There should be at least one positive control for every batch of samples tested together.
• The positive controls should be the same type of sample as that being tested (if available) i.e. blood, serum, tissues or culture supernatants.
• The control is processed in exactly the same way as the real samples.
• Ideally, the positive control virus should be of a distinctive type such that the amplicon derived from it can readily be distinguished by sequencing from that likely to be found in a sample.

Internal controls
Internal controls are strongly recommended by e.g. OIE guidelines. So far, there is no binding recommendation for the internal control.

Interpretation of results
Any test that gives rise to incorrect results for negative or positive controls should be considered invalid. Wherever possible, positive results should be confirmed by repeating the RT-PCR test. In addition, an alternative assay such as virus isolation or serology should be carried out. Where necessary, additional sample materials should be obtained.
In case of a positive PCR result, where applicable, the identity of the PCR products should be confirmed by sequencing; see appropriate chapter.
In general the analysis of index cases, i.e. suspicions of CSFV should never be performed based on one method only.
References


Laboratory test for the detection of CSF antibodies

The virus neutralization test (VNT)

Principle of test
The test is based on the determination of the neutralizing 50 % endpoint. Therefore, a constant amount of CSF virus, 100 (plus/minus 0.5 log10) tissue culture infectious doses 50 (TCID$_{50}$), is incubated with diluted serum for one hour at 37 °C. For screening purposes, the sera are initially diluted 1/5. At least two wells of PK-15 cell cultures in 96-well microplates are inoculated with mixtures of virus and diluted serum at each dilution. The plates are incubated at 37 °C for 72 hours. After this incubation period, the cell cultures are fixed and the viral antigen is detected by an immune labelling system. Either the neutralization peroxidase-linked antibody (NPLA) or the neutralization-immunofluorescence (NIF) assays may be used. The results are expressed as the reciprocal of the initial serum dilution at which half of the inoculated cell cultures fail to show any specific labelling (no viral replication detectable in the cell culture). A point between two dilution levels is estimated. The peroxidase system has the advantage that the results can be read with a light microscope or even with the naked eye. For the NIF test a fluorescence microscope is needed.

CSFV strains for the NT

Stock virus
The reference strain within the European Union is still Alfort/187 (CSF 902), although it should be supplemented by virus strains of more recent genotypes, e.g. 2.3. The reference viruses can be supplied by the Community Reference Laboratory for CSF, upon request. It can be useful to include additional field virus isolates which occur in the country in question. Batches of stock virus can be produced by inoculating one day old PK-15 cell cultures in tissue culture flasks with the respective reference virus. The cultures are incubated for 3 to 4 days at 37 °C and thereafter frozen at -80 °C. After thawing, the culture fluids are clarified by centrifugation for 10 min at 778 g. The supernatants are dispensed in small (0.5-1 ml) amounts in ampoules which are labeled, packed and stored at -80 °C until use in the NT. Their titre is determined by 10 fold titration as described below.
Calculation of infectivity titres

The highest dilution of virus which infected 50% of the cell cultures is regarded as the endpoint of infectivity. This value is estimated or calculated using the method of KÄRBER (1931). Virus infectivity titres are expressed as tissue culture infectious doses (TCID<sub>50</sub>) per volume (e.g. 0.1 ml) of virus suspension.

Example of TCID<sub>50</sub>-calculation (Method of Kärber)

\[
\log \text{TCID}_{50} = L_{1.0} - \text{Lint} (S - 0.5)
\]

L<sub>1.0</sub> = Logarithm of the highest virus dilution with the reaction rate (R) = 1.0
Lint = Logarithm of the dilution interval (int)
S = Sum of reaction rates (R)
0.5 = Constant factor

<table>
<thead>
<tr>
<th>virus dilutions</th>
<th>infected wells / total wells</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>4/4</td>
<td>1.0</td>
</tr>
<tr>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>4/4</td>
<td>1.0</td>
</tr>
<tr>
<td>10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>4/4</td>
<td>1.0</td>
</tr>
<tr>
<td>10&lt;sup&gt;-3&lt;/sup&gt; (= L&lt;sub&gt;1.0&lt;/sub&gt;)</td>
<td>4/4</td>
<td>1.0</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1/4</td>
<td>0.25</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>0/4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\[ S = 1.25 \]

\[ \log \text{TCID}_{50} = -3 - 1.0 (1.25 – 0.5) \]
[\[ -3 – 0.75 \]
[\[ -3.75 \]

The virus titer is 10<sup>3.75</sup> TCID<sub>50</sub>/0.1 ml in the original virus suspension or 10<sup>4.75</sup> TCID<sub>50</sub>/ml.

**Backtitration**

A back-titration must be mounted on every occasion that a NT is carried out. It is carried out using the actual virus added to the NT plate, and covers a range of 4 log dilutions (ie 10<sup>-1</sup> to 10<sup>-4</sup>). The back titration thus acts as one of the internal quality controls. If the back titration is
outside the tolerance limits (30-300 TCID$_{50}$ per well) then the test is invalid and must be repeated.

**Calculation of dilution factor for obtaining 10$^2$ TCID$_{50}$/50µl:**
As an example consider the stock virus has a titre of 10$^{5.7}$ TCID$_{50}$/100µl. The virus dilution employed in the VNT should have a titre of 100 (10$^2$) TCID$_{50}$/50µl.

10$^{5.7}$ TCID$_{50}$/100 µl correspond to 10$^{5.4}$ TCID$_{50}$/50 µl

calculation:
10$^{5.7}$ TCID$_{50}$/100 µl is to be divided by 2 (2 is equivalent to 10$^{0.3}$) - that means:
10$^{5.7}$ divided by 10$^{0.3}$ - that means: 5.7 - 0.3 = 5.4 (10$^{5.4}$)
10$^{5.4}$ TCID$_{50}$/50 µl are to be diluted to obtain 10$^2$ TCID$_{50}$/50 µl

calculation of dilution factor:
10$^{5.4}$ is to be divided by 10$^2$ = 10$^{3.4}$ (5.4 - 2 = 3.4)

dilution factor = 1/10$^{3.4}$ - that means: approx. 1/2600

(From 1 ml stock virus containing 10$^{5.7}$ TCID$_{50}$/100 µl (or 10$^{5.4}$ TCID$_{50}$/50µl) one can make 2600 ml of virus solution containing 10$^2$ TCID$_{50}$/50 µl)

**Test procedure**

**Neutralization reaction**
- Load 80 µl cell culture medium plus 20 µl serum sample in the first row of wells of the microtitre plate to obtain the initial serum dilution of 1/5.
- Two wells per serum dilution are used.
- The remaining wells of the plate are loaded with 50 µl of medium.
- Thereafter, 50 µl of the 1/5 serum dilution are withdrawn by means of a 12 channel pipette and diluted serially two-fold. (When titration is finished each well contains 50 µl of serum-medium dilution.)
• Add 50 µl/well of test virus suspension containing 100 TCID<sub>50</sub>/50 µl and gently shake the plate. The required dilution of the test virus to obtain 100 TCID<sub>50</sub>/50 µl has to be prepared shortly before use by diluting the virus in growth medium.

• Place the plates in a moist chamber and incubate in a CO<sub>2</sub> incubator (4-5 % CO<sub>2</sub>) for 1 hour at 37 °C. (Alternatively the plates can be sealed air tight and incubated for 1 hour at 37 °C)

• Back titrate the virus dilution and incubate together with the NT plates.

• Add 50 µl/well of growth medium containing approximately 3 x 10<sup>5</sup> cells/ml and shake gently for 5-10 seconds.

• The cell suspension should be prepared during the 1 hour incubation period.

• Place the plates in a moist chamber and incubate in a CO<sub>2</sub> incubator (4-5% CO2) for 72 hours at 37 °C. Alternatively the plates can be sealed air tight and incubated for 72 hours at 37 °C.

• Discard the growth medium, fix and stain the plates as described in Chapter immune labelling for peroxidase conjugated antibodies.

The neutralisation test can be performed in Leighton tubes, following the same principle and stained with FITC conjugated antibodies.

**Controls**

Back-titration (see above) of test virus must be carried out each time to check if the virus titre was 100 TCID<sub>50</sub>/50µl. Tolerance limits of 0.5 log either way: 30-300 TCID<sub>50</sub>/50µl had been recommended in Annex I of EU Directive 80/217. However, this was a pure mathematical figure. Evaluation of neutralisation tests over a longer period and in different laboratories show, that there is no linear correlation between back-titration and antibody titre.

A CSF antibody positive reference serum sample as well as cell controls containing medium and cells have to be included in the test. Reference sera can be supplied by the Community Reference Laboratory for CSF upon request.

The test has to be repeated if the reference sera do not give the expected result and the back titration is out of the limit. The reference sera should be monitored over time using laboratory intern tracking charts.
**Evaluation / Estimation of ND50**

Serum titres are recorded as reciprocal of the highest initial dilution of sera (dilution of serum and growth medium without virus suspension) which prevented virus replication in 50% of the wells. Wells are scored as virus positive even if only one cell within the monolayer stains specifically. A point between two dilution levels is estimated. Serum titres are expressed as neutralization dilution 50% (ND50) and can be also calculated using the method of KÄRBER (1931).

Example 1: Only one of the two wells of the serum dilution of 1:10 has infected cells. In this case the neutralization titre would be 10 ND50.

Example 2: All wells up to the dilution of 1/80 are free of viral antigen while the remaining wells with serum dilutions equal and higher than 1/160 are positive. In this case the neutralization titre is estimated to be 120 ND50.

When reporting test results, especially for export tests to other countries, it is important to specify clearly that the titre is expressed as Initial Dilution, and that for Final Dilution (which is the method mostly used in America) the result should be multiplied by 2 (ie 1/10 initial, corresponds to 1/20 final). The final dilution system is based on the actual dilution of serum during the neutralization reaction, ie after addition of virus but before adding the cell suspension.

**References**


Neutralisation Test

Test Procedure

1. Titration of serum in growth medium, starting with 1/5, 2 wells/serum dilution

2. Add 50µl test virus/well, incubate 1h at 37 °C

3. Add 50µl cell culture/well, incubate 72 hours at 37 °C, CO₂

4. Discard medium, fix and stain
**Virus titration**

log 10 virus dilution

A

- 0.1 ml virus
- 0.1 ml media
- 0.1 ml 10^1
- 0.1 ml 10^2
- 0.1 ml 10^3
- 0.1 ml 10^4
- etc.

B

Transfer of 100 μl/well of virus dilution, 4 wells/dilution

Add cells, incubate and stain as described for virus neutralization test

<table>
<thead>
<tr>
<th>V1</th>
<th>V2</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="V1 Grid" /></td>
<td><img src="image" alt="V2 Grid" /></td>
</tr>
</tbody>
</table>

(infected cells are stained dark red)

**Virus titre (TCID₅₀):**

- V1: 10^6 /0.1 ml
- V2: 10^4.7 /0.1 ml

**TCID₅₀:** reciprocal of the initial virus dilution at which 50% of the wells showed virus replication. A point between two dilution levels is estimated.
Example of ND50-Calculation (Method of Kärber)

\[
\log \text{ND}_{50} = L_{1.0} \cdot L_{\text{int}} (S - 0.5)
\]

- \( L_{1.0} \) = Logarithm of the highest serum dilution with the reaction rate (R) = 1.0
- \( L_{\text{int}} \) = Logarithm of the dilution interval (int)
- \( S \) = Sum of reaction rates
- \( 0.5 \) = Constant factor

<table>
<thead>
<tr>
<th>serum dilution</th>
<th>infected wells / total wells</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 40</td>
<td>0/2</td>
<td>1.0</td>
</tr>
<tr>
<td>1 : 80</td>
<td>1/2</td>
<td>0.5</td>
</tr>
<tr>
<td>1 : 160</td>
<td>2/2</td>
<td>0.0</td>
</tr>
<tr>
<td>1 : 320</td>
<td>2/2</td>
<td>0.0</td>
</tr>
<tr>
<td>1 : 640</td>
<td>2/2</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\[ S = 1.5 \]

\[
\log \text{ND}_{50} = -1.6 - 0.3 (1.5 - 0.5) = -1.6 - 0.3 = -1.9
\]

\[
\text{ND}_{50} = 10^{1.9} = 80
\]

![Diagram of serum dilutions and infection results](image)

- S 1: 10
- S 2: 120

\( \text{ND}_{50} \) reciprocal of the highest initial dilution of the sera which prevented virus replication in 50% of the wells.

A point between two dilution levels is estimated.

(infected cells are stained dark red)
Serological differential diagnosis of CSF

A differential neutralisation test against a BVDV and BDV strain should be carried out simultaneously in order to detect and to interpret cross reaction between CSF and other pestiviruses.

Neutralisation test against BVDV:

The test procedure is the same as described for CSF. There is at present no recommended permanent cell line for the BVD neutralisation test. Some suitable cell lines are described in Chapter 1. The BVD reference strain is NADL and can be supplied from the CRL. If BVDV infections have to be ruled out, additional field strains should be used.

References:

Neutralisation test against BDV

The test procedure is the same as described for CSF. Fetal sheep thymoid cells (SFT-R) are the cell line of choice and can be supplied from the cell collection of the Friedrich-Loeffler-Institute, Island of Riems, Germany. BD reference strain Moredun, strains Frijters and 137/4 can be supplied from the CRL. To rule out BDV infections, including recent strains from the respective country can be valuable.

References
CSF antibody ELISA:

Each NSFL is in charge for the licensing procedure of different batches of commercial CSF antibody ELISAs. The CRL does not recommend a certain commercial product. At present CSF antibody ELISAs are available from:

- CEDI Diagnostics, NL
- IDEXX/Bommeli, CH
- Synbiotics, F

A panel of reference sera that might be used for the quality control as requested by Commission Decision 2002/106/EC, Chapter VII, can be supplied upon request by the CRL. The panel comprises well characterised sera from experimentally infected animals and includes sera from pigs in the early phase of CSFV infection (< 21 days post infection), sera from reconvalescent pigs (> 21 days post infection), and sera from pigs infected with ruminant pestiviruses.

Genetic Typing of CSF virus isolates

Introduction

Strains of CSFV can be classified into a number of subgroups according to their nucleotide sequence homology (Paton et al., 2000). Further discrimination can allow even small differences between isolates to be determined and compared in order to establish degrees of viral relatedness. This genetic typing can be used to support or refute hypotheses on likely routes of virus spread and this is the basis of molecular epidemiology. Reverse-transcription polymerase chain reaction (RT-PCR) amplification of CSF virus RNA followed by nucleotide sequencing is the simplest option for generating the sequence data to perform these comparisons.

A number of different regions of the CSF virus genome may be targeted for molecular epidemiological studies. However, two particular regions have been extensively studied and provide large sequence data-sets with which new isolates can be compared. These two regions lie within the 5’ non translated region (5’NTR) of the genome, and within the E2 major glycoprotein gene (Lowings et al., 1996; Greiser-Wilke et al., 1998).
A database with an automated module for phylogenetic analysis of these sequences is accessible through the home page of the CRL, on the world wide web (Greiser-Wilke et al., 2000):

http://viro08.tiho-hannover.de

Username and password can be obtained by sending an EMAIL to irene.greiser-wilke@tiho-hannover.de or crl@tiho-hannover.de

For fine discrimination between closely related virus isolates, it is recommended that at least two different genomic target regions (5’NTR and E2) are analysed to ensure that small differences in sequence are reliable indicators of genetic relationships. Where discordant results are achieved, a third region can be examined (a part of the NS5B polymerase gene, Bjorklund et al., 1999) and the sequences from all three regions analysed independently and as a single, summated data set (Stadejek et al., 1997).

**Sequencing method**

- Extract CSF virus RNA from clinical samples or from PK15 cell cultures that have been infected with low passage CSF virus.
- Perform RT-PCR to amplify targets within the 5’NCR, E2 and/or NS5B genes.
- Determine the nucleotide sequence of the product or products (150 nucleotides of the 5’NTR, 190 nucleotides of the E2 gene and 409 nt of the NS5B gene) and compare with stored sequence information held in the databases.

The primer sequences and the PCR thermoprofiles for their use are given below:

**5'NTR**

Primers used for amplification of a 421 nt fragment of CSFV:

*CSFV-UP1 Forward:* 5’ CTA GCC ATG CCC WYA GTA GG 3’ (94-113)

*CSFV-UP2 Reverse:* 5’ CAG CTT CAR YGT TGA TTG T 3’ (514-496)

PCR thermoprofile: 35 x (95°C/45sec, 50°C/1 min, 72°C/1min), 1x (72°C/5 min).

Primers for sequencing 150 nt of the above:

*CSFV/SQ-1 Forward:* 5’ AGC TCC CTG GGT GGT CTA 3’ (146-163)

*CSFV/SQ-2 Reverse:* 5’ TGT TTG CTT GTG TTG TAT A 3’ (417-399)
E2 gene
Primers used for amplification of a 671 nt fragment of E1 and E2 of CSFV:
Forward  5’   AGR CCA GAC TGG TGG CCN TAY GA  3’  (2228-2250)
Reverse  5’   TTY ACC ACT TCT GTT CTC A  3’  (2898-2880)

PCR thermoprofile: 35 x (95°C/45sec, 55°C/1 min, 72°C/1min), 1x (72°C/5 min).

Inner set (for nested PCR - same profile, and for sequencing 190 nt of the above):
Forward  5’   TCR WCA ACC AAY GAG ATA GGG 3’  (2477-2497)
Reverse  5’   CAC AGY CCR AAY CCR AAG TCA TC 3’  (2748-2726)

NS5B gene
Primers used for amplification of a 449 nt fragment of NS5B of CSFV:
S1 Forward  5’   GAC ACT AGY GCA GGC AAY AG  3’  (11138-11157)
S2 Reverse  5’   AGT GGG TTC CAG GAR TAC AT  3’  (11586-11567)

PCR thermoprofile: 35 x (94°C/1 min, 56°C/1 min, 72°C/1min), 1x (72°C/7 min).

The same primers (S1 and S2) used for sequencing 409 nt of the above:
Figures in parenthesis correspond to nucleotide positions in Alfot-187 (Ruggli et al., 1996).

Sequence Data Comparisons
The following method is recommended to ensure comparability of results.
Each virus sequence is stored as text in the microsoft notepad program from whence it can be
directly imported into the ClustalW program in order to carry out multiple sequence
alignments (Thompson et al., 1994). Once aligned, the file is converted into Phylip format and
used in the Puzzle4 program to estimate the transition – transversion (Ts-Tv) ratio of each
data set (Strimmer and von Haeseler, 1996; 1997). The data sets are next analysed by
DNAdist producing distance matrices derived from the number of nucleotide substitutions
between the sequences (Felsenstein, 1989). In DNAdist, the maximum likelihood method of
evolution is chosen and the estimated Ts/Tv ratio is set to the above-calculated values. The
output from DNAdist is then used as the input file in a neighbor- joining tree generating
method, in this case Neighbor. Since the topology of the trees becomes more complex as the
number of viruses increases, multiple jumbles (x99) should be used and the outgroup should
always be specified as the sequence of the Kanagawa virus (CSF0309). This ensures that the trees will have a similar topography each time they are drawn. The treefile generated in the Neighbor program is then used as the input for a tree drawing program, namely; drawtree or drawgram although the trees can be looked at using other programs such as Treeview (Page, 1996).

To assess the statistical reliability of the dendrograms produced, each data set is repeatedly reanalysed using the Seqboot program. As before, the phylogeny for each repeat is calculated using DNAdist and then Neighbor. Both programs are run as described above except that the multiple data sets option (M) was set to 100 to indicate how many replicates of the alignment are in the input file. The treefile from Neighbor is then used as the input file in the Consense program, again designating the outgroup as the Kanagawa sequence. The bootstrap values are then readable in a text format which can be directly correlated to the dendrogram. Values in excess of 70 % are considered to be significant (Clewley, 1999).

**Interpretation**

For the interpretation of the genetic typing of a new CSF virus isolate it is vital to have the epidemiological data available i.e. date of isolation, species (domestic pig or wild boar) place (country and area). These data are necessary for the new virus isolate as well as from those virus isolates the new one is compared with.

**References**


ANNEX of useful addresses

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National Reference Laboratories:
(page 15)

OIE Reference Laboratories:
http://www.oie.int/eng/oie/organisation/en_listeLR.htm#A130

Addresses of commercial companies:
Bio X Diagnostics
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