Equine Arteritis Virus Isolation on cell culture

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This SOP is an OIE-based method used at the EURL, all OIE - VI based methods validated and used successfully in the PT can be used for this essay.

This SOP describes the virus isolation (VI) on cell culture to detect the presence of EAV infectious particles in equine semen.

1 SAMPLE COLLECTION AND TRANSPORT

This SOP can be used to detect the EAV infectious particles in **equine semen**.

The semen sample must be part of a full collection of sperm rich do not use pre-ejaculation samples since they are often negatives for EAV and are not considered valid specimens.

Semen should be delivered on ice as soon as possible after collection (no longer than 24h). If not possible, the sample should be immediately frozen and transported to the laboratory when convenient. An insulated styrofoam shipping container with frozen ice packs usually suffices for overnight delivery.

A drop of semen is usually observed under a microscope (x100) for the presence of sperm

2 TECHNIQUE

The table below presents the different steps of the analytical process:

Analytical process	Detailed step
Sample preparation	Centrifuge the semen at 1900 rpm for 10 minutes at 4°C
Sample dilution	Dilute sample in complete MEM MEDIA supplemented with 2% FBS to get a 10^{-1}, $10^{-2,}$ and 10^{-3} fold dilutions
Virus isolation	Perform the VI on cell culture as described in chapter 2.2.4
Result analysis	Analysis of the obtained results

2.1 NEEDED MATERIALS:

Product name	Manufacturer	Reference	Packaging
MEM	Gibco	31095-052	500 ml
NEAA 100X	Gibco	11140-035	100 ml
Sodium Pyruvate	Gibco	11360-039	100 ml
Penicillin-Streptomycin	Gibco	15070-063	100ml
Hepes 1M - 100ml	Gibco	15630-056	100 ml
Fetal Bovine Serum	Gibco	16000-044	500ml
Trypsin 0.25%-EDTA	Gibco	25200-056	100 ml
Carboxy Methyl Cellulose	Sigma	C4888	/
Formalin 10% buffered	Sigma	HT501128	/
Methanol	Sigma	65543	/
Crystal Violet	Sigma	C3886 or C0775	/
PBS 10X	Biosolve	162323G5	500 ml

RK13 cells	ATCC	CCL37	/

Manufacturer, reference of the needed products are indicated as they are used by the EURL for equine diseases other than African horse sickness for African horses. Other products can be used as long as it has been previously validated by the user laboratory.

2.2 BUFFER PREPARATION :

2.2.1 Complete MEM media with 2%FBS :

- Complete 500 ml MEM media with:
  - 5 ml of NEAA 100X
  - 5 ml of Sodium pyruvate 100X
  - 5 ml of Penicillin-Streptomycin Glutamin 100X
  - 5 ml of HEPES buffer 1M
  - 10 ml of inactivated FBS

2.2.2 0.75% CMC MEDIA :

- Dissolve 3 grams of Carboxy Methyl Cellulose medium viscosity in 70 ml of H_20, swirl while adding the CMC in water, then shake
- Let the solution stand overnight at room temperature (18-25°C)
- Autoclave for 20 minutes at 121°C
- When cooled to around 60°C, add 400ml of pre-warmed MEM media
- When cooled to around 40°C, add:
  - 5 ml of NEAA 100X
  - 5 ml of Sodium pyruvate 100X
  - 5 ml of Penicillin-Streptomycin Glutamin 100X
  - 5 ml of HEPES buffer 1M
  - 10 ml of inactivated FBS
- Complete the final volume to 500 ml with MEM media
- Then store at 4°C

2.2.3 Crystal violet plaque stain:

2.2.3.1 Crystal Violet stock solution

- Prepare a stock solution by dissolving 12 g of Crystal Violet in 600 ml of methanol
- Store at room temperature (18-25°C)

2.2.3.2 Crystal violet staining solution

- Prepare the staining solution as detailed:
  - 1 part of the above stock solution
  - 9 parts 10% buffered formalin

2.2.4 EAV isolation protocol:

2.2.4.1 VI first passage:

- Spin semen sample at 1900 rpm for 10 minutes and use supernatant for VI.
- Prepare 6 T25 cm² flasks of RK-13 cells per semen to analyze, 2 for 10^{-1} dilution, 2 for 10^{-2} dilution, and 2 for 10^{-3} dilution.
- Remove media from T25 (3-6 day old RK-13 monolayers). Do not rinse.
- Inoculate in duplicate 1 ml of each semen diluted on RK13 cells.
- Incubate the flasks at 37°C with 5% CO_2 for 1 hour.
- Observe with a microscope if the diluted semen is cytotoxic for RK13 cells; if yes, note the dilution when a cytotoxic effect is observed
- Then add 10 ml of 0.75 % CMC-MEM media-2%FBS to each flask and incubate at 37°C with 5% CO_2.
- After 4 to 5 days, harvests supernatant from flasks
- Stain RK13 cell monolayers by adding 2 ml of crystal violet staining solution in each flask for 30 minutes to 1 hour, remove the stain, rinse flasks with tap water and read for CPE.
    - If bacterial contamination occurs, the semen sample can be filtered through a 0.45µm filter and the VI retested.
    - If negative or if the sample seems cytotoxic, a second passage is performed on day4 (see paragraph 2.2.4.2).
    - If positive, the supernatant is harvested from a flask showing viral activity (store at -20°C), and the supernatant is tested for the presence of virus by PCR or by a reverse VNT against known positive and negative sera.

2.2.4.2 VI second passage:

- Aspirate media from confluent 3-5 days old RK-13 cells in T25cm² flask.
- From flasks at passage #1 10^{-1} dilution, take 1 ml and add it to 2 new T25 flasks of RK-13 cells. Do the same with passage #1 10^{-2} and 10^{-3}.
- Incubate flasks at 37°C with 5% CO_2 between 1 to 2 hours (minimum 1h).
- Then add 10 ml of 0.75% CMC-MEM media to all flasks and incubate at 37°C and 5%CO_2.
- Read after 3 to 4 days and harvest media and stain flasks as described above (2.2.4.1).

2.2.4.3 VI third passage:

Follow the same protocol as for passage#2 (paragraph 2.2.4.2).

3 PREPARATION OF CONTROLS:

3.1 POSITIVE CONTROL SEMEN:

In the absence of a characterized reference material, a positive control sample may be produced to be used as a positive control of the process. The production below is described as an example:

- Mix an EAV negative equine semen and an EAV positive supernatant issued from a VI on cell culture test. The dilution of the EAV supernatant in the negative semen is depending on the amount of EAV infectious particles and should be done to get a positive using the VI test.
- Once diluted, aliquote the positive control at least 500 µl each tube.
- Freeze the positive control aliquots at ≤ - 16°C.

3.2 NEGATIVE CONTROL SEMEN:

In the absence of a characterized reference material, a mix of negative equine semen can be used as a negative control of the analytical process.

- Aliquot the negative control (at least 150µl in each tube).
- Freeze the negative control aliquots at ≤ - 16°C.

4 PREPARATION OF THE SAMPLES TO BE ANALYZED

4.1 EQUINE SEMEN:

- Centrifuge the semen at 1900 rpm for 10 minutes at 4°C, discard the pellet.
- Dilute the centrifuged semen in complete MEM MEDIA supplemented with 2% FBS. The dilutions below are described as an example:
  - 250 µl of semen in 2.25 ml of complete MEM MEDIA supplemented with 2% FBS
  - 250 µl of 10^{-1} diluted semen in 2.25 ml of complete MEM MEDIA supplemented with 2% FBS
  - 250 µl of 10^{-2} diluted semen in 2.25 ml of complete MEM MEDIA supplemented with 2% FBS
- Proceed to VI test from the three above described dilution of the semen (see paragraph 2.2.4)

4.2 EQUINE TISSUES:

- In a 5 ml tube placed on ice, add 1 g of tissue to 2 ml of chilled MEM media.
- Crush the organ.
- Add chilled MEM media to the crushed organ to obtain a final volume of 5 ml.
- Mix gently before
- Centrifuge at 1900 rpm for 10 minutes at 4°C.
- Dispense the supernatant into clean tubes and dilute it as described below :
  - 250 µl of disgregated tissue (Organ) in 2.25 ml of complete MEM MEDIA supplemented with 2% FBS

- 250 µl of 10-1 diluted crushed organ in 2.25 ml of complete MEM MEDIA supplemented with 2% FBS
- 250 µl of 10-2 diluted crushed organ in 2.25 ml of complete MEM MEDIA supplemented with 2% FBS

- Proceed to VI test using the three above described dilutions of the tissues to analyze (see paragraph 2.2.4)

5 VALIDATION AND INTERPRETATION OF RESULTS

5.1 VALIDATION OF THE ANALYTICAL PROCESS

The validation of the analysis is carried out by observing the obtained results from the positive and negative control:

- No CPE whatever the dilution of the negative control and whatever the VI passage number as shown in figure 1.
- CPE whatever the dilution of the positive control and whatever the VI passage number as shown in figure 2.

If the obtained results of positive and negative control are validated, proceed to the analysis of the presence or absence of CPE from the obtained results of samples to analyze.

If the series of analysis is not validated, and depending on the anomaly observed, all or part of the analytical process has to be redone.

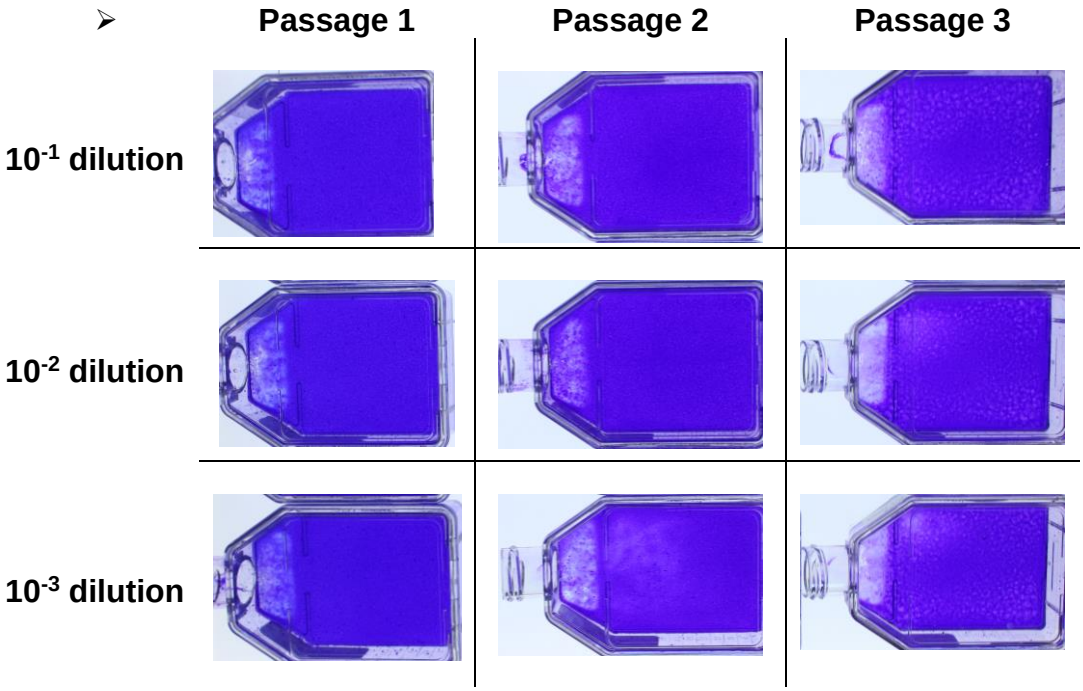

Figure 1: Example of a negative semen results according to VI on cell culture

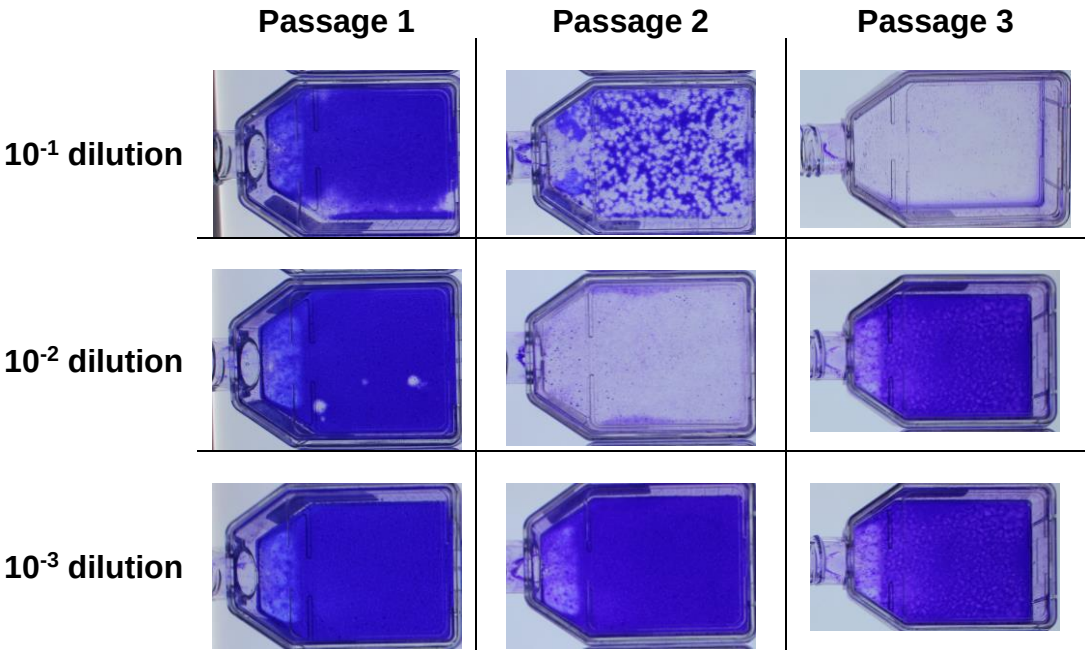

Figure 2: Example of a positive semen result according to VI on cell culture

5.2 INTERPRETATION OF RESULTS

For a semen or tissues sample, the result can be expressed as follows:

- "Positive" in the presence of CPE
- "Negative" in the absence of CPE

- To confirm that the presence of CPE is due to the infection EAV infection, the presence of EAV in the supernatant can be checked by a specific EAV RT-qPCR assay or by a reverse VNT assay against known positive and negative sera.

6 STORAGE CONDITIONS AND ELIMINATION OF SAMPLES

Store products and consumables in keeping with suppliers' recommendations.

Keep all semen samples in a refrigerated unit at (5 ± 3)°C until the test is done (not more than 24h after receipt) or in a freezer at ≤- 16°C if the storage will be longer than 24H.

EAV VI positive supernatant may be kept in a freezer at ≤ - 60°C after performing centrifugation at 4500 rpm for 10 minutes at 4°C and discarding the pellet.