VIRUS NEUTRALIZATION TEST FOR EQUINE ARTERITIS VIRUS (EAV)

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

The serological test for the diagnosis of equine arteritis virus is based on the virus neutralization test (VNT) according to the world organization for animal health (OIE) international standard: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – Chapter 3.5.10, Equine viral arteritis (infection with equine arteritis virus) (current version).

1 SAMPLE COLLECTION

1.1 SAMPLES TO BE EXAMINED

This described VNT should be performed from serum samples.

1.2 TRANSPORT OF SAMPLES

Samples (blood or serum) must be placed in a safety package and if possible, be kept cold during transport. Upon reception, the samples must to be stored at 5±3°C until analyzed.

2 TECHNIQUE

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

Analytical process	Protocol
Preparation of sample	Centrifuge sample to analyze at 2000 rpm for 10 minutes.
VNT	Perform the VNT test (paragraph 2.4)
Analysis of results	Interpretate the results using a microscope

2.1 NEEDED MATERIALS:

Product	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
Guinea Pig Complement	TEBU-BIO (Rockland)	039C300-0010	10 ml
Trypsin 0.25%-EDTA	Gibco	25200-056	100 ml
PBS 10X	Biosolve	162323G5	500 ml
RK13 cells	ATCC	CCL37	/
EAV from Bucyrus strain	ATCC	VR796	/

Manufacturers, references for 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 CULTURE MEDIA PREPARATION

2.2.1 Complete MEM

In 500 ml MEM medium, add:

- 5 ml of Non Essential Amino-Acid (NEAA) 100X
- 5 ml of Sodium pyruvate 100X
- 5 ml of Penicillin-Streptomycin-Glutamine 100X
- 12.5 ml of Hepes 1M

2.2.2 Complete MEM 10% FBS

In 500 ml of complete MEM (described above paragraph 2.2.1), add:

- 50 ml of inactivated FBS

2.2.3 Complete MEM with 10% guinea-pig (GP)

In 500 ml of complete MEM (described above paragraph 2.2.1), add:

- 50 ml of GP

2.3 EAV VIRAL SOLUTION

2.3.1 Viral stock production

The 50% Tissue Culture Infective Dose (TCID50) of the used EAV batch in the VNT should be higher than 10^5. The production below is described as an example:

- Use a T150cm² flask of RK-13 cells with a monolayer of cells covering 80% of the surface
- Discard the medium and inoculate 1 ml of the reference EAV (Bucyrus strain) to the flask
- Incubate for 1h at 37±2°C in a humid atmosphere of 5% CO_2 in air (shake the flask gently after the first 30 minutes)
- Add 30 ml of complete MEM with 10% FBS and incubate until a viral cytopathic effect (CPE) is observed.
- Collect the supernatant in 50 ml tubes and clarified it of cellular debris by low-speed centrifugation at 2000 rpm for 10 minutes
- Collect the supernatant and filter it through a 0.45µm filter
- Dispense 1 ml aliquots into cryogenic vials and store them at ≤ - 65°C

2.3.2 Viral stock titration

- Make serial tenfold dilutions from 10^{-1} to 10^{-8} of the viral stock solution in complete MEM. Each dilution are tested in 10 replicates.
- Distribute 50 µL of complete MEM in wells of a 96-well microplate
- Add 50 µl of each viral dilution in 10 wells

- Incubate the plates for 1 h at 37±2°C in a humid atmosphere of 5% CO_2 in the air
- Dispense 100 µl of RK-13 cells solution from 3- to 5-day-old at a concentration between 1.5 and $2x10^5$ cells/ml in complete MEM with 10% FBS
- Check cell viability by doing at least 4 cells control wells:
  - add 100 µl of complete MEM then 100 µl of RK-13 cells solution as described above
- Shake the plates gently and incubate for 72 h at 37±2°C in a humid atmosphere of 5% CO_2.
- Read the plates microscopically for viral CPE and calculate the virus titer using the Spearman–Kärber method
- Define the dilution to apply on the viral stock solution to obtain a viral working solution ranged from 30 to 300 TCID50 in VNT. Usually, 200 TCID50 is targeted.

2.4 VNT PROTOCOL :

- Inactivate sera at 56°C for 30°minutes in a water bath
- In a 96-well plate, add 50 µl of complete MEM to each well
- In the first row, add 50 µl in duplicate of each serum to be tested
- After mixing the first row by pipetting, take out 50 µl and made serial twofold dilutions until the last row with a multichannel pipette
- Add 50 µl of stock virus made up to contain from 100 to 300 TCID50 diluted in complete MEM with 10% GP to all wells **except** the test serum toxicity control wells and cell control wells (see paragraph 2.4.1).
- A virus back titration of the working dilution of stock virus is included, using ten wells per tenfold dilution.
- Shake the plate gently and incubate for 1 h at 37±2°C in a humid atmosphere of 5% CO_2.
- Dispense 100µl of 1.5 and $2x10^5$/ml of RK-13 cells in complete MEM with 10% FBS in all wells.
- Gently shake the plates and incubate for 72h at 37±2°C in a humid atmosphere of 5% CO_2.
- Read the plates microscopically for nonviral CPE after 12-24 hours to detect cytotoxic sera and again for viral CPE after 48-72 hours' incubation.
- Read the plates microscopically after 48-72 hours' incubation to detect CPE or no CPE in each wells.

2.4.1 VNT controls :

1.1.1.1. Serum toxicity control

- Add 75µl of complete MEM
- Distribute 25µl of serum in duplicate.
- Shake the plate gently and incubate for 1 h at 37°C±2°C in a humid atmosphere of 5% CO_2.
- Dispense 100µl of 1.5 and $2x10^5$/ml of RK-13 cells in complete MEM with 10% FBS.

- Gently shake the plates and incubate for 72h at 37±2°C in a humid atmosphere of 5% CO_2.

1.1.1.2. Cells control

- Add 100 µl of complete MEM in quadruplicate at least.
- Dispense 100 µl of RK-13 cells solution from 3- to 5-day-old at a concentration between 1.5 and $2x10^5$ cells/ml in complete MEM with 10% FBS
- Gently shake the plates and incubate for 72h at 37±2°C in a humid atmosphere of 5% CO_2.

1.1.1.3. Virus Control

Titre of the working viral solution used for the VNT needs to be checked in each test as follows:

- Make a serial tenfold dilution from 10^{-1} to 10^{-3} of the working viral solution.
- Perform the titration of these tenfold dilutions as described in paragraph 2.3.2.

3 PREPARATION OF CONTROL:

3.1 POSITIVE CONTROL SERUM:

It's possible to get characterized reference materials, as positive reference sera to EAV produced by the EURL for equine diseases. A positive control sample may be also produced to be used as positive control to validate the analytical process. The production below is described as an example:

- Dilute a positive serum to EAV in a known negative serum
- Once diluted, aliquote the positive control
- Freeze the positive control aliquots at ≤ - 16°C

3.2 NEGATIVE SERUM

A negative control serum can be obtained using a serum known as be negative to EAV.

Positive and negative sera are available and can be provided by the EURL.

4 VALIDATION AND INTERPRETATION OF RESULTS

4.1 VALIDATION OF THE ANALYTICAL PROCESS

The validity of the test is confirmed by establishing that the:

- cells control wells have nonviral CPE
- the obtained titre of the working viral solution is ranged from 30 to 300 TCID50/50µl. To calculate titre of the virus see paragraph 4.1.1
- positive serum controls are in accordance of the expected titre plus or minus one dilution
- negative serum control is conform

4.1.1 Spearman-Kärber method

According to the Spearman-Kärber formula: Log_{10} Median Dose = $(X_0 - (d/2) + d(\sum r_i/n_i)$

where: X_0 = $\log_{10}$ of the reciprocal of the lowest dilution at which all test inocula are positive.
d = $\log_{10}$ of the dilution factor (i.e. the difference between the log dilution intervals)
n_i = number of test inocula used at each individual dilution (after discounting accidental losses)
r_i = number of positive test inocula (out of n_i).
$\sum(r_i/n_i)$ = $\sum(P)$ = sum of the proportion of positive tests beginning at the lowest dilution showing 100% positive result.

Summation is started at dilution X_0.

Example I: EAV titration - ***Equal Numbers of wells per dilution***

Log10dilution	n_i	r_i
- 3	10	10
- 4	10	10
- 5	10	10
- 6	10	10
- 7	10	7
- 8	10	3
- 9	10	1
- 10	10	0

$P = r_i/n_i$ = proportion of positive tests (i.e. affected inoculum wells) at each individual dilution.

Log_{10} $TCID_{50}$ per volume inoculated (50µl)	=	7.6
Therefore, EAV doses per 50µl is	=	$10^{7.6}$

4.2 RESULT INTERPRETATION:

As mentioned in the OIE terrestrial manual, chapter 3.5.10:

The serum exhibited antibodies to EAV if *"A serum dilution is considered to be positive if there is an estimated 75% or greater reduction in the amount of viral CPE in the serum test wells compared with that present in the wells of the lowest virus control dilution. End-points are then calculated using the Spearman–Kärber method. A titre of 1/4 or greater is considered to be positive.*

A serum is considered to be negative if *"only have a trace (less than 25%) or no virus neutralization at the lowest dilution tested."*

According to the result interpretation, serum results can be expressed as above:

- Positive with the obtained serum titre of antibodies to EAV,
- Negative,
- Cytotoxic.

5 STORAGE CONDITIONS AND ELIMINATION OF SAMPLES

Store products and consumables in keeping with suppliers' recommendations.

Keep all control sera under frozen conditions at ≤ - 16°C

After analysis, all the samples should be discarded following the rules applied by the lab performing this test.