DETECTION OF ANTIBODIES AGAINST ORBIVIRUS BY SERONEUTRALIZATION TEST (SNT)

Date: 22/06/2021	Document code: GL-LCV-09	*Rev. 02*

1. SCOPE

To detect the presence of neutralizing antibodies (serotype specific) against Orbivirus (African horse sickness virus, Bluetongue virus and Epizootic haemorrhagic disease virus) in serum samples of animals.

2. MATERIALS AND EQUIPMENT

Material and reagents

Disposable pipette sterile tips with filter (100, 200 and 10 µl range)

Sterile 96-well flat bottom plates

Dilution plates with "U" bottom (optional)

Graduated disposable pipettes (1,5 and 10 ml)

Containers for dispensing reagents

Container biosanitary waste

Disinfectant

Sterile 1,5 ml conical Eppendorf tube

Sterile tubes of 10 and 50 ml

EMEM: Eagle's Minimum Essential Medium with Earle's balanced salt solution.

Glutamine

Antibiotic-antimicotic

Non-essential amino acids

EDTA Trypsin (0,25%)

Inactivated bovine foetal serum (IBFS).

Positive serum control: hyperimmune serum with known titre

Negative serum control: sera without antibodies against the serotype analysed.

Stock Virus, previously growth and full titrated

Vero cell suspension

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Equipment

Laminar Flow Cabin Type II

Stove incubator 37 +/- 2ºC and 5% CO_2

Inverse Phase Contrast Microscope

Vortex (stirrer)

Thermostatic bath (56 +/- 2ºC)

Ultra Freezer (<-60 ° C)

Refrigerator (2 - 8º C)

Freezer (<-18ºC and> -60ºC)

Single-channel micropipettes in ranges 2 - 20µl, 10 - 100µl, 100 - 1000µl

Multi-channel micropipettes of ranges: 5 - 50µl, 30 - 300µl

Automatic pipettor

Chronometer

Microcentrifuge

Haemocytometer

3. METHOD

The assay measures the ability of a serum to neutralize a fixed dose of virus and prevent the appearance of a readily observable cytopathic effect (CPE) when both are co-inoculated in susceptible cells culture. SNT is considered a semi-quantitative assay and an antibody titre is reported together with the qualitative result (POS/NEG).

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4. PROCEDURE

PREPARATION OF THE ASSAY

Each Orbivirus serotype tested is considered as an individual assay. For that reason, it is necessary to include all the controls (positive and negative serum control, cell control and back titration virus control), except the sample cytotoxicity control, for each serotype.

For each assay, samples and sample cytotoxicity control will be included in SAMPLE PLATES and the remaining controls will be included in a CONTROL PLATE (see annex 1).

Depending on the purpose of the analysis and the number of samples to be analysed, chose the proper protocol:

A/ Screening: to analyse a large number of samples. The main purpose is to know if the samples are positive or negative (qualitative results). Then, 3 or 4 two-fold dilutions of serum are included and at least two samples in duplicate in each test, such as quality control. Therefore, 22 to 24 samples could be tested per plate

B/ Titration: there will be seven or eight dilutions and at least two replicates of each sample, therefore a maximum of 6 samples per plate could be tested.

Calculate the number of plates needed to test the samples in the chosen protocol (screening or titration). Calculate the final working dilution virus volume (Vf) needed depending on the number of plates needed and considering that 25 µl virus / well is added and each plate have 96 wells (~100 wells).

Vf (µl) = 25 µl/well x 100 wells/plate x n plates

Calculate the cell suspension volume (Vcell) needed depending on the number of plates

Vcell (µl) = 50 µl/well x 100 wells/plate x n plates

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Prepare the serum samples to test according to the Guidelines for SNT; including the incubation of the serum samples in a bath (56-±2ºC) for 30 ± 5 min. to inactivate the fixation complement system.

PREPARATION OF REAGENTS

- Diluent: EMEM (Eagle's Minimum Essential Medium) + 1% Antibiotic/Antimycotic (100x) + 1% L-Glutamine + 1% Non - essential Aminoacids.
- Cell culture medium: diluent supplemented with 10% of inactivated bovine foetal serum
- Cell suspension:
  - Remove the cells from a Vero flasks using trypsin and resuspend the cells in an appropriate amount of diluent.
  - Using an appropriate haemocytometer (i.e. *Neubauer* counting chamber) and a suitable dye exclusion method to count the viable cells.
  - Prepare the calculated volume of cell suspension in cell culture medium containing 2 x 10^5 viable cells/ml.
- Working dilution of virus (WD):

  Considering the chemical formula: **Ci x Vi = Cf x Vf**

  Vi = Vf x (Cf/Ci)

  Where,

  Ci= titre of the stock virus - $TCID_{50}$ / 25µl

  Vi= volume of the stock virus necessary to prepare the WD (unknown) - µl

  Cf= final concentration of WD - 100 $TCID_{50}$/ 25µl

  Vf= final volume of working dilution virus (see Preparation of assay) - µl

  Add Vi volume of the stock virus on (Vf-Vi) volume of diluent to get the WD.

PERFORMANCE OF THE ASSAY

A. Diluent and sera (samples and controls)

- Prepare a sterile 50 ml tube with (Vf-Vi) volume of diluent and label it as WD

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- Prepare 3 sterile "Eppendorf" tubes with 900 µl of diluent and label them 10, 1 and 0,1

SAMPLE PLATES

Screening protocol:

- Add 45µl of diluent to the rows A and E; 40µl to the rows B and F; and 25µl to the remaining rows.
- Add 5µl of serum samples to the rows A and E (serum control)
- Add 10µl of serum samples to the rows B and F, mix it up and transfer 25µl to the rows C and G; mix it up and transfer 25µl to the rows D and H. Mix it up and discard 25µl.

Titration protocol:

- Add 45µl of diluent to the row A; 40µl to the rows B; and 25µl to the remaining rows.
- Add 5µl of serum samples to the row A (serum control)
- Add 10µl of serum samples to the row B, mix it up and transfer 25µl to the row C; continue the 2-fold serial dilution until row H. Mix it up and discard 25µl.

CONTROL PLATE

- Add 25 µl of diluent to the columns 1 to 4 (back titration), columns 9 and 10 rows B-H (positive control serum) and columns 11 and 12 rows B-D (negative control serum)
- Add 40 µl of diluent to the columns 9 and 10 row A (positive control serum)
- Add 40 µl of diluent to the columns 11 and 12 row A (negative control serum)
- Add 50 µl of diluent to the columns 11 and 12 rows E-H (cell control)
- Add 10µl of positive control serum to the columns 9 and 10 row A, mix it up and transfer 25µl to the row B; continue the 2-fold serial dilution until row H. Mix it up and discard 25µl.
- Add 10µl of negative control serum to the columns 11 and 12 row A, mix it up and transfer 25µl to the row B; continue the 2-fold serial dilution until row D. Mix it up and discard 25µl.

B. Virus control and neutralizing

- Add the Vi volume of the stock virus into the sterile 50 ml tube with (Vf-Vi) volume of diluent labelled as WD.

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- Shake and transfer 100 µl of WD of virus to the "Eppendorf" tubes with 900 µl of diluent, labelled as 10. Continue the 10-fold dilutions until 0,1.

CONTROL PLATE

- Add 25 µl of control virus labelled as 0,1 to column 4
- Add 25 µl of control virus labelled as 1 to columns 3
- Add 25 µl of control virus labelled as 10 to column 2
- Add 25 µl of WD virus to column 1
- Add 25 µl of WD virus to the wells containing the positive and negative serum controls

SAMPLE PLATES

- Add 25 µl of WD virus to all wells on the plates, except to the wells containing the sample cytotoxicity control.

- Incubate for 1 hour at 37 ± 2 ° C in the presence of 5% CO_2.

C. Addition of cells

- Add to all wells in all plates 50 µl / well of suspension cells containing 2 x 10^5 viable cells/ml
- Incubate during five days at 37 ± 2 ° C in the presence of 5% CO_2

D. Microscope Reading

- Use an inverted phase contrast microscope by using the 4X (and 10X if necessary) viewer to read the plates and observe presence / absence of CPE (Annex 3).
- In the CONTROL PLATE result sheet, mark with “e” each well where CPE is observed. Cell control should be marked as “OK” if there is an optimum cell growth.
- In the SAMPLE PLATES result sheet, mark with a “-”the first dilution of each serum sample where CPE is observed. Sample cytotoxicity control should be marked as “OK” if there is an optimum cell growth.

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5. ANALYSIS AND INTERPRETATION OF RESULTS

The test is accepted if:

- No CPE is observed in any of the wells in Cell Control
- Estimated backtitration (virus control) applying the Reed and Muench method should be between 30 and 316 $TCID_{50}$ / 25 µl. This means that:
    - CPE is observed in all the "100" and "10" dose wells
    - No CPE is observed in none of the wells containing "0,1" doses
    - Number of wells where CPE is observed in wells of the row containing the "1" dose, can be between none (30 $TCID_{50}$/25 µl) to all wells (316 $TCID_{50}$/25 µl).

  NOTE: Reed and Muench method allows CPE in wells containing "0,1" doses, if there are the same number of wells without CPE in the "1" dose row.
- Negative control serum should be Negative (<1/5). CPE would be observed from the first serum dilution.
- Positive control serum should be Positive. Estimated antibody titre, applying the Spearman-Karber method would have the expected titre, accepting as valid a dilution above or below.

If the assay fails to meet all the acceptance criteria listed then review the assay for any technical errors and repeat the assay for whatever serotype(s) that failed.

Repeatability control: samples analysed in replicate must have the same qualitative result. In case of positive samples, it would be accepted as valid a dilution of difference among replicates

The **sample citotoxicity control wells** should show a correct cell growth. If cytotoxicity or contamination was observed, the sample result could be reported only if the reading of the sample is undoubted. If tested sample shows cytotoxicity or it is contaminated, apply next criteria:

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- If tested serum is cytotoxic or contaminated at one or more dilutions and CPE is observed at the first dilution after the one that produces toxicity or contamination; serum must be reported as cytotoxic or contaminated (examples a and b).
- If tested serum is toxic or contaminated at one or more dilutions and CPE is not observed at the next dilutions after the one that produces toxicity or contamination; serum must be reported as positive and the titre (examples c and d).

Examples:

	a	b	c	d
1/5	Toxic	Cont	Cont	Toxic
1/10	Toxic	(-)	Cont	
1/20	Toxic		Cont	
1/40	(-)		Cont	(-)
1/80				
1/160				
1/320			(-)	
1/640				
RESULT:	Toxic	Cont	1/160	1/20

(-) First dilution where CPE is observed

Interpretation of Results

The presence of CPE from the first dilution sample (1/5) indicates that the serum does not contain specific neutralizing antibodies, so that the virus has not been neutralized and consequently has produced lysis on cells. The sample will be considered as **NEGATIVE** (<1/5).

The absence of CPE in sample wells indicates that the serum contains neutralizing antibodies specific to that serotype, so that the virus has been neutralized. The sample will be considered as **POSITIVE** and the titre will correspond to the last dilution of the serum sample where the CPE is not observed.

The result is expressed in antibody titre, for example: POS 1/40 (expressed in log 1,60)

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6. ANNEXES

ANEXX 1: *ORBIVIRUS* SERONEUTRALIZATION TEST SUMMARY

ANEXX 2: EXAMPLE OF THE PREPARATION OF WD VIRUS

ANEXX 3: CPE in the framework of this SOP

7. REFERENCES

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Chapter: Bluetongue (Infection with Bluetongue virus)
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ANEXX 1 *ORBIVIRUS* SERONEUTRALIZATION TEST SUMMARY

1. SAMPLE PLATES

Screening protocol (example including Sample citotoxicity control)

		1	2	3	4	5	6	7	8	9	10	11	12
Serum control	A	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ
1/5	B	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ
1/10	C	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
1/20	D	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
Serum control	E	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ
1/5	F	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ
1/10	G	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
1/20	H	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl

25 µl

25 µl

At least two samples in duplicate

Titration (example including 3 replicates and Sample cytotoxicity control)

		SAMPLE 1			SAMPLE 2			SAMPLE 3			SAMPLE 4		
		1	2	3	4	5	6	7	8	9	10	11	12
Serum control	A	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ	5µ+45µ
1/5	B	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ
1/10	C	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
1/20	D	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
1/40	E	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
1/80	F	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
1/160	G	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
1/320	H	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl

25 µl

2. CONTROL PLATE

										C+	C+	C-	C-
	1	2	3	4	5	6	7	8	9	10	11	12
A	25µ+25	25µ+25	25µ+25	25µ+25					10µ+40µ	10µ+40µ	10µ+40µ	10µ+40µ
B	25µ+25	25µ+25	25µ+25	25µ+25					25 µl	25 µl	25 µl	25 µl
C	25µ+25	25µ+25	25µ+25	25µ+25					25 µl	25 µl	25 µl	25 µl
D	25µ+25	25µ+25	25µ+25	25µ+25					25 µl	25 µl	25 µl	25 µl
E	25µ+25	25µ+25	25µ+25	25µ+25					25 µl	25 µl	50 µl	50 µl
F	25µ+25	25µ+25	25µ+25	25µ+25					25 µl	25 µl	50 µl	50 µl
G	25µ+25	25µ+25	25µ+25	25µ+25					25 µl	25 µl	50 µl	50 µl
H	25µ+25	25µ+25	25µ+25	25µ+25					25 µl	25 µl	50 µl	50 µl
	100	10	1	0,1							CC	CC

25 µl

VIRUS CONTROL (BACKTITRATION)

Two-fold dilution

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ANEXX 2 EXAMPLE OF THE PREPARATION OF WD VIRUS

We have to analyse **18 samples** using a **titration protocol** (i.e. samples in duplicate). Therefore we need **4 plates** (3 Sample plates and 1 Control Plate)

Final volume of WD virus is **Vf = 10ml = 10000µl** (*Vf = 25 µl/well x 100 wells/plate x n plates*)

If the titre of the stock virus is **10^6 $TCID_{50}$/ml = $2{,}5x10^4$ $TCID_{50}$/25µl (Ci)**

Considering the chemical formula: **Ci x Vi = Cf x Vf**

Ci x Vi = Cf x Vf

Vi = Vf x (Cf/Ci)

Where,

Ci= titre of the stock virus - $TCID_{50}$ / 25µl

Vi= volume of the stock virus necessary to prepare the WD (**unknown**) - µl

Cf= final concentration of WD - 100 $TCID_{50}$/ 25µl

Vf= final volume of working dilution virus (see point 4.1) - µl

Vi = 10000 x (100 / 25000) = 40 µl

Therefore, 40 µl of the stock virus should be added on (10000 – 40 = 9960 µl) of diluent to get the WD virus

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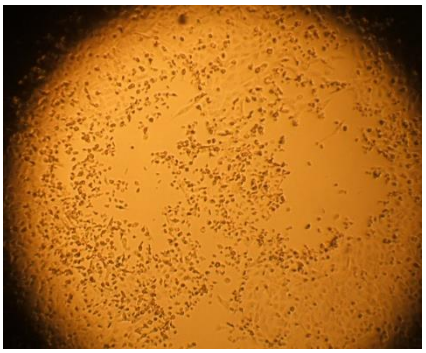
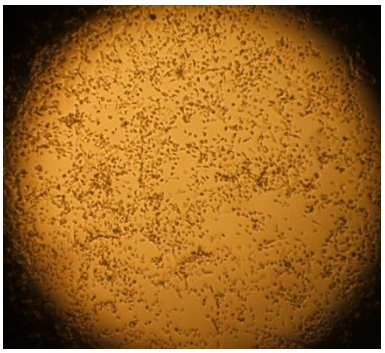
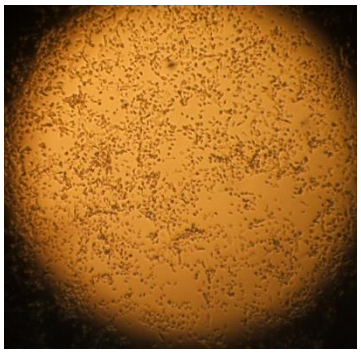
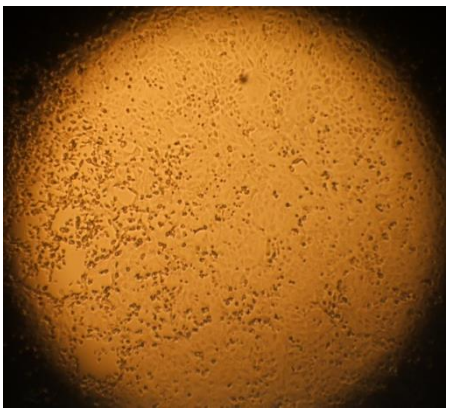
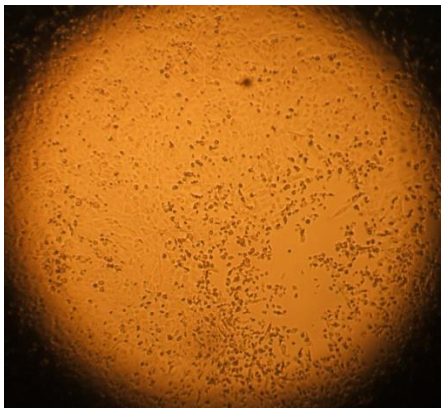
ANEXX 3 CPE in the framework of this SOP

CPE will be considered when more than 10% of the cells in well are affected.

Examples of CPE:

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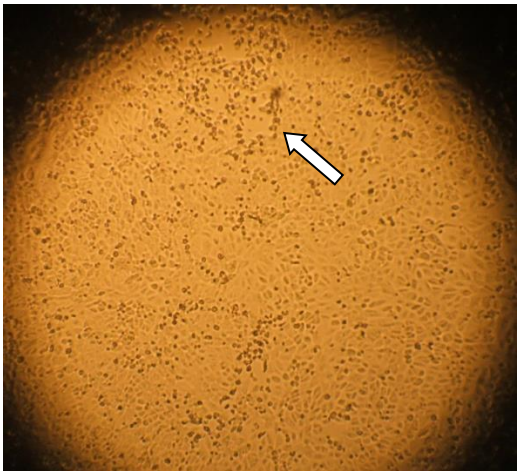
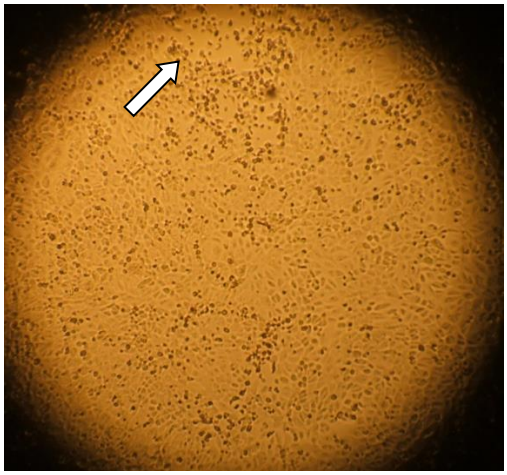
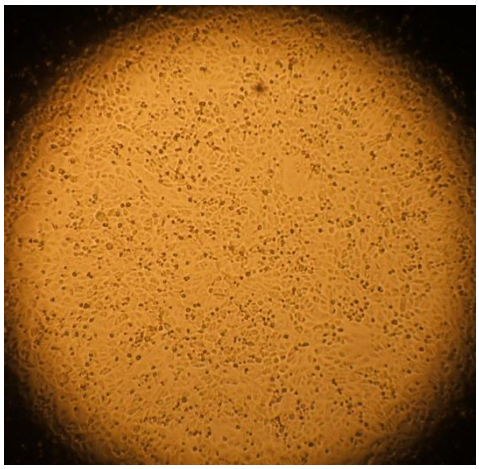
Examples of no CPE (less than 10%): when less than 10% of the cells in the well are affected. It is not consider as CPE in the framework of this SOP.





Example of no CPE observed:



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