STANDARD OPERATING PROCEDURE

Detection and titration of West Nile Virus specific antibodies using virus neutralization methods

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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.

This standard operating procedure (SOP) is used for the serological diagnosis of West Nile virus (WNV) infection, in case of clinical suspicion of West Nile (WN) infection in horses. It describes virus neutralization techniques, and in particular the OIE gold standard plaque reduction neutralization test at a 90% plaque reduction level.

Detection of neutralizing antibodies by Plaque reduction neutralization test is described in the Manual on Diagnostic Tests and Vaccines for Terrestrial Animal, 2018 edition (English version) of the Office International des Epizooties (OIE), Chapter 3.1.24.

Virus neutralization tests rely on the *in vitro* inhibition of WNV cell infections, in the presence of anti-WNV neutralizing antibodies in the tested sample.

Two methods can be used to evaluate cytopathogenic effects (CPE) induced after contact of Vero cells with West Nile virus: observation of lysis plaques in 6-well plates (PRNT, plaque reduction neutralization test) or observation of infected and refringent cells in 96-well plates (microneutralization test).
If specific anti-WNV antibodies are present in the sample, they will neutralize infectious viral particles and protect cells from infection.

1. SAFETY

Class2 biosafety cabinet : WNV is assigned to Biosafety Level 3 (BSL3). WNV is a human pathogen which can cause severe or life-threatening illness. Users have to follow the rules applicable to the handling of infectious materials and waste: work in a secured level 3 biosafety laboratory is mandatory when infectious WNV is manipulated. Process samples that may potentially contain live agents in an approved biological safety cabinet with HEPA filtration. All surfaces and equipment that come into contact with infected materials must be disinfected with an appropriate disinfectant and virucide (i.e. Anios for example). All contaminated instruments, containers and fluids must be autoclaved before reuse or disposal.

2. COLLECTION OF SAMPLES

2.1 TISSUE MATERIAL TO BE EXAMINED

The serological diagnosis of WNV is performed on serum from horses. Blood must be collected in a tube without anticoagulants. The whole blood samples can be subjected to a low speed centrifugation (780g for 10 min) for serum portion separation. The serum must not be haemolysed or coagulated.

2.2 TRANSPORT OF SAMPLES

Sera can be transported under positive (5°C ± 3°C) or negative (≤-16°C) cold conditions.

3. MATERIALS, EQUIPMENTS AND REAGENTS

3.1 MATERIALS AND EQUIPMENTS

- Vortex
- Water baths at + 40 °C (± 1 °C) and at + 56 °C (± 1 °C)
- Micropipets, either single channel or multichannel (for the microneutralization test)
- Sterile tips with filters, adapted to micropipets
- Pipet-aid
- Sterile pipettes
- Microscope
- CO_2 incubator at + 37 °C (± 1 °C), 5% CO_2
- 6-well sterile culture plates (PRNT)
- 96-well sterile culture plates, flat-bottom (Microneutralization test)
- 1.5 ml sterile eppendorf tubes (PRNT)

3.2 REAGENTS

- Vero cells (kidney epithelial cells from African green monkeys), a mammalian cell line very sensitive to West Nile virus (Origin : Institut Pasteur)
- Culture medium : DMEM + 5% Fetal Calf serum (Lonza) + 1% sodium pyruvate + 1% penicillin/streptomycin (InVitrogen)
- West Nile Virus, strain IS-98-ST1: produced in Vero cells (Origin: Institut Pasteur).
- Animal serum samples, as well as positive and negative reference sera (of known titer). Aliquotes of reference sera are stored frozen at < – 16 °C. Decomplement sera at 56°C for 30 minutes.

PRNT method:

- Agarose SeaPlaque (Cambrex, reference : 50100) : prepare 4% SeaPlaque agarose in sterile milliQ water. Storage temperature: + 5 ° C (± 3 ° C)
- MEM 2X (InVitrogen). Storage temperature: + 5 ° C (± 3 ° C)
- Purple cristal stock solution (to be protected from light): weigh 2g of purple crystal and dissolve in 10 ml of absolute ethanol. Prepare the working solution by diluting 2 ml of the stock solution, 12 ml of paraformaldehyde at 32% and 86 ml distilled water. Store at room temperature for 2 months.

4. PROCEDURE

Important: serum samples need to be decomplemented at + 56 °C (± 1 °C) for 30 min prior to their use in virus neutralization tests.

4.1. PRNT METHOD

Day-1:

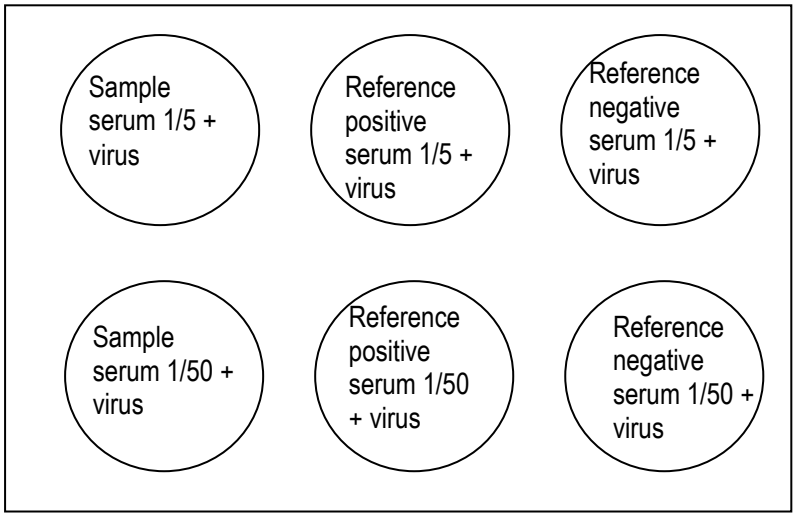
- Trypsin Vero cells and resuspend the cell pellet in 10ml of culture medium.
- Count cells (prepare a 1/10th dilution and use trypan blue exclusion to consider only viable cells).
- Distribute 7,5x10^5 cells per well of a 6-well plate under 2 ml of culture medium.

Day 0:

- Prepare 300µL of dilutions of sample or reference sera in DMEM, in 1.5 ml eppendorf tubes: for each serum (sample or reference), prepare 1/5 and 1/50 dilutions (refer to the plate map below)

To prepare the 1/5 dilution: mix 90µl serum + 360µl DMEM. For the 1/50 dilution, dilute 30µl of the 1/5 dilution in 270µl DMEM.

- Prepare two additional controls for every analysis round : one cell control with DMEM only (600µL) and one virus control containing 300µL DMEM
- Prepare a virus suspension containing 100 lysis plaques/250µl DMEM (400 lysis plaques/ml DMEM) and add 300µl of this virus suspension in every eppendorf tubes, except for the cell control tube. Due to the mixing of equal volumes of serum and virus, final dilutions of the sera are 1/10 (for the 1/5 initial dilution) and 1/100 (for the 1/50 initial dilution).
- Incubate for 90 min in a CO_2 incubator at 37 °C (± 1 °C).
  Discard the culture medium of every wells, and add 0.5 ml of the virus-serum mix or 0.5ml of DMEM (cell control).



Add the following controls also=

Virus control: 500µL DMEM containing 100pfu of WNV

Cell control: 500µL DMEM

- Incubate 90 minutes in a 5% CO_2 incubator at 37 °C (± 1 °C).
- During adsorption, melt the sterile 4% Seaplaque agarose and incubate it at 42°C. Prepare 2X complete MEM (10% fetal calf serum, 2% sodium pyruvate, 2% penicillin/streptomycin) and incubate it at 40°C. Equal volumes of 4% Seaplaque agarose and complete 2X MEM solutions are adjusted to 40°C and mixed together just before use (prepare 30mL mixed solution for a 6-well plate). Incubate at 40°C.
- Discard the virus suspension in contact with cells and add 4 ml of the agarose medium per well. Let it solidify at room temperature for 15 min and incubate for 3 days in a CO_2 incubator at 37 °C (± 1 °C).

Day 3:

- After a 72h incubation of infected cells, add 1 ml of the purple crystal working solution and incubate for 24h at 37°C. Very gently discard the agarose layer and wash 3 times with PBS.
- Count lysis plaques for each condition (should be approximately 100 plaques in the virus control; for the test to be valid, should be comprised between 80 and 120 plaques).

Endpoints are based on a 90% reduction in the number of plaques compared with the virus control.
The serum is considered positive if in corresponding wells, less than 10% of the plaques counted in the virus control (less than 10 plaques in general) are observed. The animal serum titer corresponds to the opposite of its latest dilution, for which less that 10 plaques are observed. The serum is considered negative if less than 90% reduction in the number of plaques is observed.

4.2. MICRONEUTRALIZATION TEST:

- Add 50µL of DMEM in every wells of a 96-well plate, except for lines A and B and columns 11 and 12 (used for titration of the virus suspension). Wells in line H will be used as virus controls or cell controls.

- Dilute sera in duplicate in 96 well-plates (factor 2 serial dilutions, starting 1/5th to 1/160): Add 40µL of DMEM and 10µL of samples or reference serum in line A, add 80µL of DMEM and 20µL of sample or reference serum in line B (see the plate map).
- In lines B to G, prepare serum dilutions. Add 50µl of the previous dilution (previous line) to 50µl of DMEM (throw out 50µl of medium in wells of line G after dilution).

Plate map:

	Negative serum		Positive serum		Serum 1		Serum 2		Serum 3		Virus titration	
A	1/5 – cytotoxicity control										10^{-1}	10^{-2}
B	1/5										10^{-1}	10^{-2}
C	1/10										10^{-1}	10^{-2}
D	1/20										10^{-1}	10^{-2}
E	1/40										10^{-3}	10^{-4}
F	1/80										10^{-3}	10^{-4}
G	1/160										10^{-3}	10^{-4}
H	Virus contr	Virus contr	Virus contr	Virus contr	Virus contr	Cell contr	Cell contr	Cell contr	Cell contr	Cell contr	10^{-3}	10^{-4}

- Addition of virus: prepare a virus suspension at 100 $TCID_{50}$/50µL.
- Add 50µL of this suspension in every wells of columns 1 to 10, except for wells H6-10 (cell controls) and wells A1-10 (controls of the serum cytotoxicity) in which 50µL DMEM are added.
- Wells « Virus Titration »: dilute the virus suspension used for the Microneutralization test at 1/10, 1/100, 1/1.000 and 1/10.000 Dispatch 50µL of each dilution in 4 subsequent wells. Add 50 µl of DMEM in the 16 wells used for titration.
- Incubate the plates at 37°C for 1h30min.
- Add Vero cells: Add trypsin to a 75 cm^2 Vero culture flask and resuspend cells in 10ml of complete DMEM medium. Count cells using a 1/10th dilution of the cell suspension and prepare a cell suspension at $2x10^5$ cells/ml. Add 100µl of this cell suspension per wells.
- Incubate the plate at 37°C for 3 days.
- Read under the microscope: observe wells for absence or presence of CPE (CPE = no or damaged cells). In virus controls, CPE must be present, whereas in cell controls, no CPE are found. The titration part should give the following result: CPE in 4 wells at 10^{-1} dilution, CPE in 1-3 wells (2 on average) at 10^{-2} dilution (if not, the virus titer is incorrect and the test will be performed again).

A result is considered positive if in wells incubated with animal sera, no CPE is observed. The animal serum titer corresponds to the opposite of its latest dilution, for which no CPE is observed. Serum titers are comprised between 10 and 320 after multiplication of initial dilution factors by a factor of 2; sera are indeed diluted by a factor of 2 during virus-serum mixing.

5. VALIDATION AND INTERPRETATION OF RESULTS

5.1. VALIDATION OF THE TEST

PRNT METHOD

- No viral plaque should be present in the cell control : 0 plaque in the cell control
- Count viral plaques in the virus controls : the assay is valid if between 80 and 120 viral plaques are present
- No protective effect is visualized with the negative reference serum, eg approximately the same number of viral plaques are counted in the virus control and in the wells with the negative reference serum, whatever its dilution was.
- The positive reference serum protects Vero cells from infection

MICRONEUTRALIZATION TEST:

- Absence of infected cells in the cell controls
- Presence of infected cells in the virus controls
- Presence of an intact cell monolayer in line A (control for the cytotoxic effects of the animal sera); if the cell monolayer is destroyed, the corresponding serum should be tested again and will be considered as cytotoxic; a new serum sample is needed to observe the WNV neutralization capacity of the serum.
- Virus titration: the assay is valid if all the wells at 10^{-1} and 2 on average (between 1 and 3) at 10^{-2} are infected.
- No protective effect is visualized with the negative reference serum, eg every wells with the negative reference sera are infected.
- The positive reference serum protects Vero cells from infection

5.2. INTERPRETATION

- If cells are infected (microneutralization test) or if the same number of viral plaques is counted (PRNT method), whatever the serum concentration is considered: the serum is negative
- If cells are protected with the first serum dilutions (microneutralization test) or if less than 10% of the viral plaques counted in the virus control are counted with the first serum dilutions (PRNT method): the serum is positive and its titer is the inverse of the latest dilution at which cells are protected or at which less than 10% of the viral plaques counted in the virus control are observed

6. STORAGE CONDITIONS

Whenever possible, t is preferable for all blood samples to be centrifuged and stored without the clot. For long-lasting storage, it is advisable to freeze sera without clot at ≤ -16°C.
Before use, and for maximum 1 week after use, the serum samples are stored at + 5 ° C (± 3 ° C). Beyond that delay, they are stored at ≤- 16 ° C.