Virus neutralization test for detection of anti-capripox virus neutralizing antibodies

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1. Purpose and Scope

This Standard Operating Procedure describes the use of the virus neutralization assay (VNT) for the detection in serum or plasma from cattle, sheep, goats or other susceptible species, of neutralizing antibodies against capripox viruses (CAPX) including lumpy skin disease virus (LSDV), sheep pox virus (SPPV) and goat pox virus (GTPV).
This SOP describes how the CAPX VNT is performed, from sampling and preservation to preparation of the reagents, performing the test, interpretation and validation of the results and removing and discarding the reagents and the samples.

2. Definitions et abbreviations

- AEC: 3-amino-9-diethyl-carbazone
- BSL: Biosafety Level
- CAPX: capripox
- DMEM : Dulbecco's modified Eagle's medium
- EDTA: Ethylenediaminetetraacetic acid
- FBS: Foetal Bovine serum
- GTPV: goatpox virus
- H2O2: hydrogen peroxide
- LSDV: lumpy skin disease virus
- NA: not applicable
- OA3.Ts: ovine testis cell line
- PBS: Phosphate buffered saline
- PBST: Phosphate buffered saline + 0,05% Tween20
- SOP: standard operating procedure
- SPPV: sheeppox virus
- TCID50: Median Tissue Culture Infectious Dose
- VNT : Virus neutralization test

3. Principle of the method

This virus neutralization test is performed with a dilution of serum titrated against a constant titer of Capripox virus. The different dilutions of decomplemented sera are diluted in DMEM 2% and incubated in 96-well plates with a constant dilution of capripox virus (LSDV in case of LSDV-VNT, SPPV in case of SPPV-VNT or GTPV in case of GTPV-VNT) for 1 hour. OA3Ts cells are added to each well and the plates are incubated for 4 days. On day 4 post infection, supernatant is removed and plates are frozen. Cell are then fixed with paraformaldehyde and then methanol/peroxide. A fixed dilution of a positive control serum is then added to the fixed cells in duplicate. Following a wash step to remove unbound antibodies, a secondary antibody, conjugated to horseradish peroxidase is added. Finally, following a wash step to remove unbound secondary antibody, 3-amino-9-diethyl-carbazole is added as a substrate for the peroxidase. The staining reaction is then stopped by eliminating the substrate and adding Na-acetate buffer.

If neutralizing anti-capripox antibodies are present in the test sera, they bind to the virus inhibiting infection of the cells, subsequent immune-staining of the cells will reveal presence or absence of infection foci when the plates are analyzed by inverted microscopy.

If no neutralizing anti-capripox antibodies are present in the test sera, the virus can infect the cells and infection foci will be observed when the wells are analyzed by inverted light microscopy.

If neutralization occurs, the unbound antibodies are washed away, leaving the peroxidase-coupled secondary antibody without a target to bind to. No infection foci will be observed when the wells are analyzed by inverted light microscopy.

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4. Safety, biosafety and environment

Wear gloves. Avoid skin/eye contact with paraformaldehyde, methanol, H_2O_2, 3-amino-9-diethyl-carbazole, and Na-acetate. No pipetting by mouth, only mechanical pipetting.

The test is performed under BSL-3 conditions, at least until the cells and virus are fixed. Inactivated control serum can be added to fixed plates under BSL-2 conditions.

5. Sample collection

5.1. Sample collection

Serum samples are collected according to standard procedures for aseptic blood sampling by a veterinarian or FELASA-certified staff. Before use, the serum samples are decomplemented at 56°C for 30 min.

5.3. Transport of the samples

Samples should be packed and transported according to the Triple Packaging Principle.

6. Experimental procedure

6.1. Consumables

6.1.1. Reagents

- DMEM high glucose, pyruvate
- Foetal bovine serum
- Gentamicine
- Amphotericin B
- Trypsine-EDTA
- OA3Ts cells
- Citric acid (pH2)
- Virocid (or isopropanol)
- PBS
- Paraformaldehyde
- Methanol
- H_2O_2
- Skim milk powder
- Tween 20
- Anti-Bovine IgG-peroxidase or Anti-Sheep IgG-peroxidase or Anti-Goat IgG-peroxidase
- Sodium acetate
- AEC (3-amino-9-diethyl-carbazone)
-

6.1.2. Lab equipment

- Disposable gloves
- Sterile disposable tips for micropipetts
- Sterile serological pipets (for use with pipette controller (Pipetboy)
- Sterile 96-well microplate (flat bottom wells)
- Sterile 15 ml tubes
- Sterile 15 ml tubes
- Sterile Culture flasks
- Timer
- Micropipettes
- Pipet controller
- Flameboy + cartridge

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- beaker
- Vortex
- Hemocytometer (or equivalent)
- Incubator 37°C, 5% CO2
- Warm water bath at 37 °C
- Biosafety cabinet
- Table top centrifuge
- Refrigerator (4°C)
- Freezer (-20°C)
- Freezer (-80°C)
- Inversion light microscope

6.1.3. Preparation of solutions

- 4% paraformaldehyde
- 30:1 Methanol/30%H_2O_2
- Blocking buffer
- PBST
- Sodium acetate buffer
- Substrate (3-amino-9diethyl-carbazone in NA-acetate, 0.05% H_2O_2)
- Positive control serum

6.1.4. Preparation of culture media

- OA3Ts Medium :
  - DMEM high glucose, pyruvate
  - FBS (10%)
  - Gentamicine 1µg/ml
  - Amphotericin B 20 µg/ml

6.1.5. Preparation of the test serum samples

- A serie of 8 decomplemented serum samples are diluted in DMEM 2%:
  - 1/2
  - 1/10
  - 1/50
  - 1/250
  - 1/1250
  - 1/6250
  - 1/31250
  - 1/156250

6.1.6. Preparation of virus dilution

- Prepare 1 dilution of the virus:
  - TCID100

6.1. Method

6.1.7. Infection of OA3Ts

Add 50 µl of each dilution of test serum in twofold to the wells of a sterile flat-bottom 96-well plate. On each plate a positive control is run (no serum, only virus dilutions and cells) in 6 wells, and a negative control is run in 6 wells (no serum or virus, only cells). Depending on the capripox species, prepare LSDV, SPPV or GTPV dilution and add 50 µl to the wells containing test serum and incubate 1h at 37°C and 5% CO_2.

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Meanwhile prepare the OA3Ts cells and add 100 µl to each well on the 96-well plate. Plates are then left to incubate for 4 days at 37°C and 5% CO_2

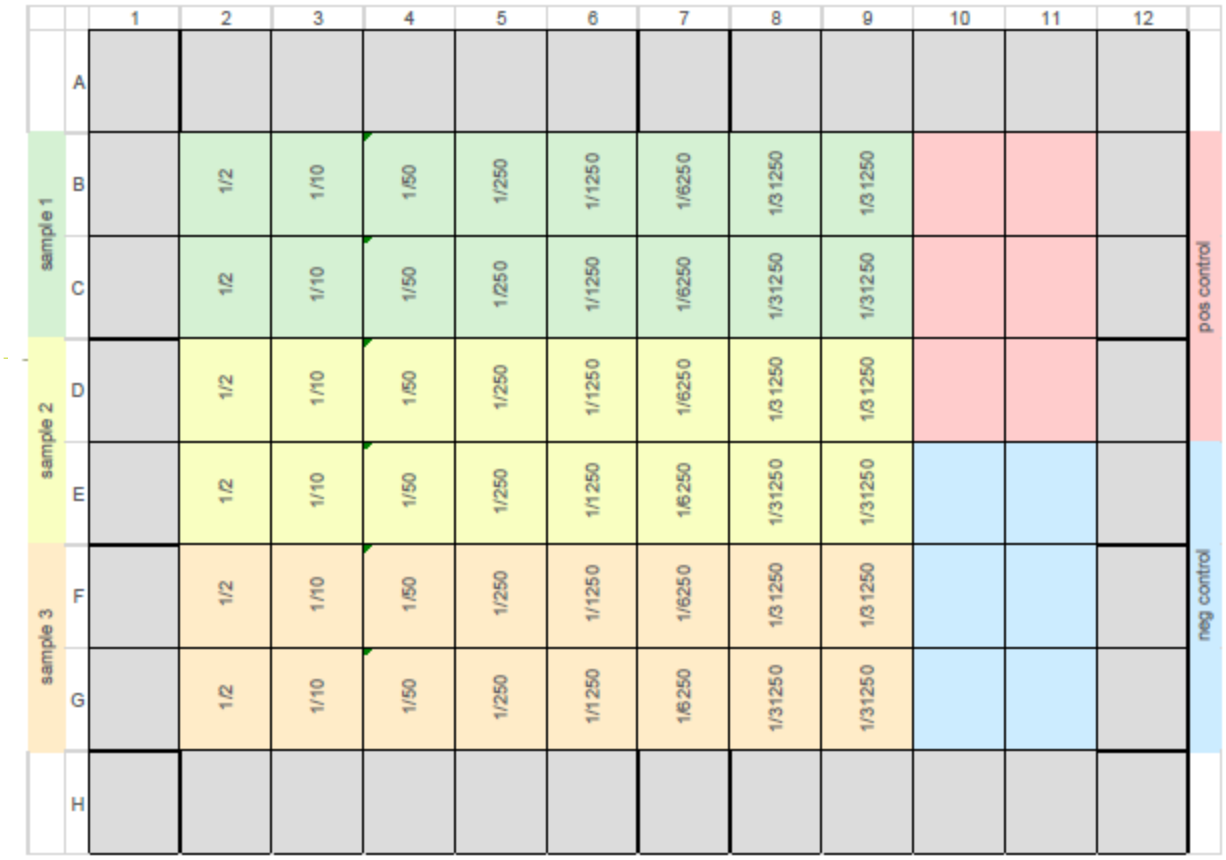

Figure 1:Plate set-up for 3 samples. Each sample is diluted as mentioned and added to the plate in duplicate. Positive controls (cells + virus) and negative controls (cells only) are added in 6-fold.

6.1.8. Fixation of infected cells

Following 4 days of incubation, the supernatant is removed by pipetting 200 µl growth medium from each well with a multichannel pipette and wells are washed twice with 100 µl PBS. Plates are left to dry for 60 +/- 6min at 37°C and then frozen at -80°C for min 30min.

Cells are fixed by addition of 4% para-formaldehyde (diluted in PBS, 100 µl/well) for 10+/-1 minutes. Supernatant is removed and cells are washed twice with PBS for minimum 5 minutes. One hundred microliters of freshly prepared methanol/H_2O_2 (30:1) is added to each well and incubated for 5 +/-0.5 minutes before twice washing with PBS for 5 minutes.

6.1.9. Staining

The positive control serum (primary antibody) is diluted 1/400 in PBSmilk (1%) and 50 µl is added to the fixed cells. Plates are then incubated for 60 +/-6 minutes at 37°C. Following the incubation, plates are washed twice with 300 µl of PBST (0.05% Tween20), before the secondary antibody is added. To each well 50 µl of a 1:1000 dilution in PBST of the horseradish peroxidase coupled secondary antibody (Anti-Bovine IgG-peroxidase or Anti-Sheep IgG-peroxidase or Anti-Goat IgG-peroxidase) is added to each well and plates are incubated for 60 +/-6 minutes at 37°C.

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Following the incubation, plates are washed twice with 300 µl PBST and 50 µl AEC substrate is added and left to incubate for 15 to 20min at room temperature. The substrate is then removed from the plates and 100 µl of stop solution (sodium-acetate buffer) is added to each well.

6.1.10. Reading of the plates

Plates are read twice, once within 30 min of having added the stop solution and once more 24h after addition of the stop solution. Under an inversion light microscope each well is analyzed for the presence or absence of stained foci.
Results are calculated as the mean value of the highest concentration where no foci are seen and the lowest concentration were foci are seen. If the mean value is equal to 1/50 or lower (for example 1/3750), the sample is positive. If the mean value is higher than 1/50 (For example 1/30), then the sample is considered negative.

6.1.11. Acceptance criteria

The results are valid only if:

- Negative controls remain foci free
- Positive controls are positive for foci

Depending on the purpose of testing, the anamnesis etc. the Technical Responsible may advice to re-test the sample for confirmation of the result.

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