Immunoperoxidase monolayer assay for the detection of anti-capripox virus antibodies

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1. Purpose and Scope

This Standard Operating Procedure describes the use of the immunoperoxidase monolayer assay (IPMA) for the detection in serum or plasma from cattle, sheep, goats or other susceptible species, of 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 IPMA 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
- GTPV: goatpox virus
- H2O2: hydrogen peroxide
- IPMA: Immunoperoxidase monolayer assay
- 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

3. Principle of the method

OA3.Ts cells are seeded in 96-well plates. Once confluency is reached the cells are infected with 100 $TCID_{50}$ of capripox virus (LSDV in case of LSDV-IPMA, SPPV in case of SPPV-IPMA or GTPV in case of GTPV-IPMA). Following 3 days incubation at 37°C, supernatant is removed and plates are frozen. Cell are then fixed with paraformaldehyde and then methanol/peroxide. Dilutions of test serum are 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 anti-capripox antibodies are present in the test serum, they bind to the virus on the surface of the infected cells, addition of the peroxidase-coupled secondary antibody and subsequent substrate will reveal infection foci when the plates are analyzed by inverted microscopy.

If no anti-capripox antibodies are present in the test serum, 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 plates are analyzed by inverted light microscopy.

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4. Safety, biosafety and environment

Wear gloves. Avoid skin/eye contact with paraformaldehyde, methanol, H2O2, 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 test sera can be added to fixed plates under BSL-2 conditions. Disposable material is discarded in autoclavable trash bag.

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.

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

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- Pipet controller
- Flameboy + cartridge
- 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. Method

6.1.5. Infection of OA3Ts

Prepare 96-well plates to be confluent within 24h (10^4 OA3Ts cells/well). Depending on the capripox species, prepare LSDV, SPPV or GTPV dilutions to add 100 $TCID_{50}$ to each well and incubate 72h at 37°C and 5% CO_2.
A backtitration of the virus used for the IPMA is included. The IPMA is only valid if the backtitration reveals an infection with 30 to 300 $TCID_{50}$ per well.

6.1.6. Fixation of infected cells

Following 72h incubation, the supernatant is removed by pipetting 100 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.

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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/H2O2 (30:1) is added to each well and incubated for 5+/-0.5 minutes before twice washing with PBS for 5 minutes.

6.1.7. Addition of test sera

Each serum is diluted in PBSmilk (1%) and 50 µl is added in duplicate to the fixed cells. At the very least dilution 1:50 and 1:300 are used. Alternatively a full dilution series can be used if a precise antibody titer is needed. A positive and a negative control serum are also added to 2 wells on each plate. Plates are then incubated for 60+/-6minutes at 37°C.

6.1.8. Staining

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+/-6minutes at 37°C.

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. Plates are read twice, once within 30 min of having added the stop solution and once more 24h after addition of the stop solution.

6.1.9. 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 qualitative: positive if foci are observed, negative if no foci can be observed.
If an antibody titer is needed, this can be calculated as follows:

$$t = (1/d).10^{r(n/N + ½)}$$

t = titer
d = greatest serum dilution that results in all wells positive
r = logarithmic difference between dilutions
n = 0.5 if wells contain <50% foci, 1 if wells contain >50% foci
N = number of replicates (here 2)

6.1.10. Acceptance criteria

The results are valid only if:

- Uninfected cells remain foci free
- Infected cells incubated with negative control serum are foci free
- Infected cells incubated with positive control serum are positive for foci.
- The backtitration of the virus used indicates 30 to 300 TCID50 were added to each well.

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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