Virus isolation of capripoxviruses using the OA3.Ts cell-line

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1. Purpose and scope

This procedure describes the isolation of capripoxviruses (Lumpy Skin disease and sheep- and goatpox). This method can be used on blood samples and organ tissue of cloven-hoofed animals that need to be investigated for the presence of capripoxviruses. The isolated viruses will be detected by IPMA and/or stored for further use.

2. Definitions and abbreviations

AB	antibiotic
AEC	3-amino-9-diethyl-carbazone
ATCC	American Type Culture Collection
CO_2	carbon dioxide

CPE	cytopathic effect
DMEM	Dulbecco's modified eagle medium
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
H_2O_2	Hydrogen peroxide
IPMA	immunoperoxidase monolayer assay
LSD	lumpy skin disease
MDBK	Madin–Darby bovine kidney
OA3.Ts	ovine testis cell line
PBS	phosphate buffered saline
PBST	0.05% Tween20 in PBS
SOP	standard operating procedure

3. Principle of the method

To isolate capripoxvirus, the OA3.Ts cell line (ATCC® CRL6546™) will be inoculated with tissue suspensions or blood originating from infected cloven hoofed animals. Detection of viral growth will be done using the IPMA (Immunoperoxidase monolayer assay).

4. Safety, biosafety and environment

Work must be done within biosafety confinement level 3, at least until the cells have been fixed. All operations take place in the laminar flow cabinet where the work space is continuously ventilated with sterile air. The lab technician must always wear gloves and pipetting is done mechanically. The work surface is disinfected with a suitable disinfectant such as Virocid™. Each time a flask is opened or closed, a flame is passed over the opening using a Flameboy™.
Do not work with capripoxvirus in the laminar flow cabinet of the clean cell culture room.

5. Experimental

5.1. Consumables

5.1.1. Reagents

- AB: Gentamicine (1µg/ml) and Amphotericin B (20 µg/ml)
- AEC (3-amino-9-diethyl-carbazone)
- Anti-Bovine IgG-peroxidase or Anti-Sheep IgG-peroxidase or Anti-Goat IgG-peroxidase
- Citric acid (pH2)
- DMEM high glucose, pyruvate, Store at 5°C (±3)
- FBS
- H_2O_2
- Methanol

- OA3.Ts cells[1] (ATCC® CRL6546™): Fetal ovine testis cell line. Store in liquid nitrogen <-130°C
- Paraformaldehyde
- PBS: Stored at 5°C (±3)
- Skim milk powder
- Sodium acetate
- Sterile water
- Trypsin-EDTA
- Tween 20
- Virocid™

5.1.2. Solutions

4% paraformaldehyde

- 30:1 Methanol/30%H2O2
- Blocking buffer (PBS/milk (1%))
- PBST
- Stopping solution (Sodium acetate buffer)
- Substrate (3-amino-9diethyl-carbazone in Na-acetate, 0.05% H2O2)
- Positive control serum

5.1.3. Preparation of culture media

OA3.Ts Medium:

- DMEM high glucose, pyruvate
- FBS (10%)[2]
- Gentamicine 1µg/ml
- Amphotericin B 20µg/ml

5.1.4. Lab equipment

- Beads for organ/tissue homogenization
- Biosafety cabinet 2
- Cryopreservation tubes
- Disposable gloves
- Eppendorf tubes (1,5 ml)
- Flameboy™ (+ gas fillings)
- Freezer (-20°C)
- Freezer (-80°C)
- Hemocytometer (or equivalent)
- Incubater 37°C, 5% CO2
- Inversion light microscope
- Liquid waste recipient and solid waste recipients

[1] MDBK cells (ATCC® CCL-22™) can be an alternative for the OA3.Ts cells. These cells are adult Bos Taurus kidney cells. Both OA3.Ts and MDBK are continuous cell lines.
[2] DMEM + 10% FBS is used to culture the OA3.Ts cells. The FBS concentration is lowered to 2% when virus is cultured.

- Micropipets, Pipet controller
- Refrigerator (4°C)
- Sterile 15 ml tubes
- Sterile 50 ml tubes
- Sterile Culture flasks (25 cm^2)
- Sterile disposable filter-tips for micropipetts
- Sterile serological pipets (for use with pipette controller)
- Tissue lyser
- Tabletop centrifuge
- Timer
- Vortex
- Warm water bath at 37 °C

5.2. Method

5.2.1. Preparation of cell culture

For the preparation of the cell culture of OA3.Ts cells, see SOP cell culture[3].

5.2.2. Preparation of the samples

Starting from organ/tissue: Add 80 mg of the organ/tissue to 1 ml PBS in an Eppendorf tube. Add beads to the Eppendorf tube. If possible, prepare multiple Eppendorf tubes. Place the Eppendorf in a tissue lyser and lyse for 6 minutes at 25 Hz.[4] Harvest the supernatant after the lysis and filter using a 0,45 µm filter.

Starting from blood: Add 250 µl of blood to 1 ml sterile PBS in an Eppendorf tube (washing of the blood). Centrifuge 2 minutes at 2000 rpm and remove the supernatant. Add 1 ml of sterile water (lysis) to the pellet. Dilute in 4 ml PBS + AB.

5.2.3. Infection of the cells

Starting from organ/tissue: Add 500 µl of the suspension to the OA3.Ts cells in a 25cm^2 bottle. The OA3.Ts cells need to be 80-90% confluent. Add 500 µl of DMEM +2% FBS + AB to the cells and incubate for 1 hour at 37°C and 5% CO_2. Then add 9 ml DMEM + 2% FBS + AB and incubate for one week at 37°C and 5%CO_2.

Starting from blood: Add 1 ml to the OA3.Ts cells in a 25 cm^2 bottle and 1 ml of DMEM +2% FBS + AB. The cells must be 80-90% confluent. Incubate for 30 min at 37°C and 5% CO_2. Add 9 ml DMEM +2%FBS + AB and incubate for one week at 37°C and 5%CO_2.

A positive (using reference virus) and a negative control (using PBS) flask can be included.

[3] Based on the ATTC manual for OA3.ts cells
[4] Or use an alternative method for tissue homogenization

5.2.4. Detection via IPMA

Although capripoxvirus-induced CPE can be observed, the CPE is not always clearly visible, especially with LSD virus. The confirmation of the results of the virus isolation can be done by IPMA. Usually within 6 to 14 days from inoculation.

If possible prepare several flasks in parallel so when one flask is harvested and stained using IPMA on day 7 post inoculation, a parallel flask can be kept in culture a few days longer.

if you want to keep de produced virus, harvest the supernatant from the flask and alliqote into cryopreservation tubes to be stored in liquid nitrogen.

5.2.4.1. Fixation of infected cells

Following 7 day incubation, the supernatant is removed (alliquoted and stored) and cells are washed twice with 2 ml 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, 2ml/flask) for 10+/-1 minutes. Supernatant is removed and cells are washed twice with 2 ml PBS for minimum 5 minutes. 2 ml of freshly prepared methanol/H_2O_2 (30:1) is added to the flask and incubated for 5+/-0.5 minutes before twice washing with PBS for 5 minutes.

5.2.4.2. Addition of test sera

The reference serum is diluted[5] in PBS/milk (1%) and 1 ml is added to the fixed cells. flasks are then incubated for 60+/-6minutes at 37°C.

5.2.4.3. Staining

The reference serum is diluted5 in PBS/milk (1%) and 1 ml is added to the fixed cells. flasks are then incubated for 60+/-6minutes at 37°C.

Following the incubation, flasks are washed twice with 2 ml of PBST (0.05% Tween20), before the secondary antibody is added. To each flask 1 ml 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 flask and flasks are incubated for 60+/-6minutes at 37°C.

Following the incubation, plates are washed twice with 2 ml PBST and 1 ml AEC substrate is added and left to incubate for 15 to 20 min at room temperature. The substrate is then removed from the plates and 2 ml of stop solution (sodium-acetate buffer) is added to each well.

5.2.4.4. Reading of the flasks

Flasks 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 flask 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.

[5] The optimal dilution of the reference serum is to be determined in advance.