Viral isolation and identification of African swine fever virus in peripheral monocytes

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1. Virus isolation and identification

1.1 MATERIALS AND REAGENTS

- Donor animal: domestic pig or wild boar (Sus scrofa).
- Mechanical defibrinator.
- Adsorbent paper.
- Stopwatch.
- 50 ml tubes.
- CO2 incubator (±0.5%)/ 37±3ºC.
- Counter chamber [THOMA or NEUBAHUER or similar characteristics].
- Eppendorf tubes of 1.5ml.
- Freezer <-10 ºC.
- Freezer <-70 ºC.
- Refrigerator 4±3 ºC.
- Glass or plastic pipettes for volumes of 1-25 ml.
- Latex or nitrile gloves.
- Class II laminar flow cabinet.
- 96-well flat-bottom cell culture plate [NUCLONTM Surface, NUNC or similar characteristics].
- Multichannel pipette of 50-300 l.
- Inverted phase contrast cell culture microscope.
- pH meter (0.01 UpH).
- Pipetboy acu or equivalent.
- Incubator with shaker 37±2ºC.
- 250 ml and 500 ml sterile glass bottles.
- Single-channel pipettes 1-10 µl.
- Single-channel pipettes 10-100 µl.
- Single-channel pipettes 10-200 µl.
- Single-channel pipettes 200-1000µl.

- Tabletop centrifuge with 4ºC cooling, Megafuge 1.0R [Heraeus rotor #7570 or similar].
- Vortex.
- Ammonium chloride NH4Cl [Ref.: 1.01145.1000 (Merck) or similar characteristics]. Store at 4ºC.
- Phosphate buffered saline solution (PBS 1x) in tablets [Ref.: 524650-1 (CALBIOCHEM) or similar characteristics]. Store at room temperature.
- Türk's dye. [Ref.: 1.09277.0500 (Merck) or similar characteristics]. Store at room temperature.
- Positive control: ASF virus positive ADH isolate. Store at ≤ -70°C.

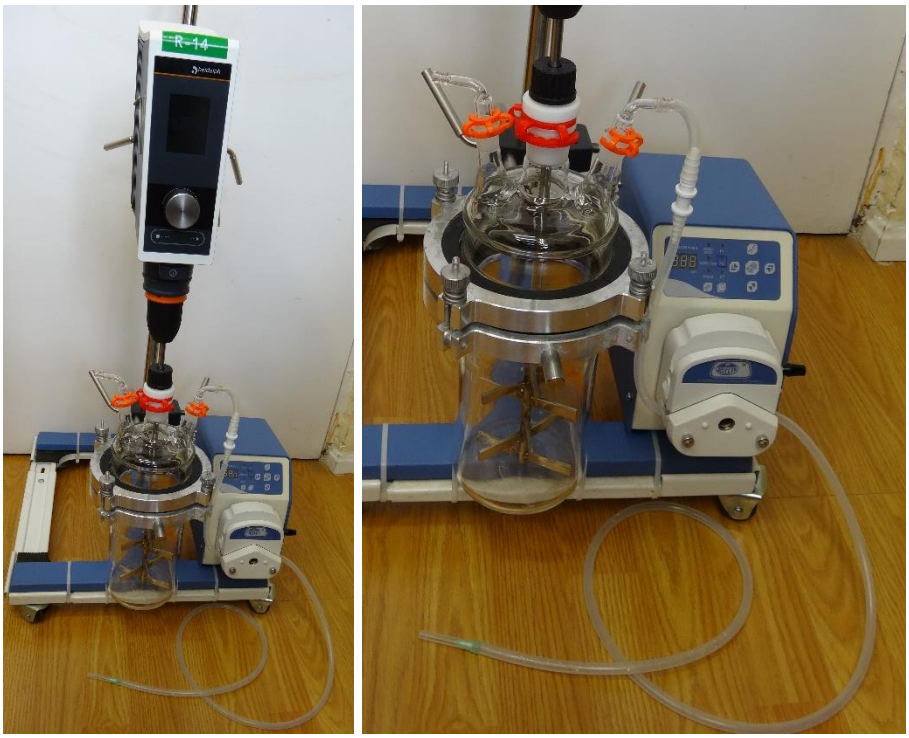

Figure 1. Mechanical defibrinator.

1.2 METHODOLOGY:

1.2.1 EXTRACTION AND CULTURE OF LEUKOCYTES:

- Obtaining defibrinated blood from a donor pig or wild boar:
  - Sedate the animal.
  - Before the blood extraction, introduce the needle with which the extraction will be performed into the tube that connects to the defibrinator, previously started (Figure 1).
  - Perform the blood extraction through the ophthalmic venous sinus.
  - Once the blood collection has been completed, leave the blood inside the defibrillator for 30 minutes with the blades rotating.

- Plate preparation:

  - ***Reagents:***
    - *Erythrocyte lysis solution:* Ammonium chloride NH4Cl, sterile at 0.83% (8.3gr of NH4Cl in 1 liter of distilled water).
    - *Phosphate buffered saline solution: (PBS 1X, pH ±2):* Tablets can be used directly (1 tablet in 1 liter of distilled water) or manufactured as follows:

      ClNa- ---------- 8gr
      ClK ---------- 0.2gr
      PO_4H_2K ---------- 0.2gr
      PO_4HNa_2 ---------- 1.15gr
      Distilled water ---------- 1 litro

  - Discard the fibrin clot generated by the defibrinator.
  - Aliquot the blood in the defibrinator in 50ml sterile tubes in a laminar flow cabinet.
  - Centrifuge for 30 minutes at 2,500rpm, without brake.
  - We will obtain three phases: serum (culture medium for leukocytes), a thin white layer of leukocytes and a third fraction of erythrocytes.
  - Transfer the serum to a sterile 250 ml jar and use it in the last step as culture medium.
  - Transfer the erythrocytes to a 50 ml tube and store at 4ºC.
  - Transfer the leukocytes to a 50 ml tube and add 3 volumes of erythrocyte lysis solution.
  - Incubate on ice for 15 minutes.
  - Centrifuge at 2,000 rpm for 15 minutes, with brake.
  - Discard the supernatant, carefully and add, again, 3 volumes of erythrocyte lysis solution.

- Incubate on ice for 15 minutes.
- Centrifuge at 2,000 rpm for 15 minutes, with brake.
- Discard the supernatant. If the pellet still contains erythrocytes, perform one or two washes with 1X PBS (centrifugations will be at 2,000rpm for 10 minutes).
- Resuspend the leukocyte pellet with 10ml of the previously collected serum.
- Perform the leukocyte count and adjust the leukocyte suspension to a final concentration 107 leukocytes/ml, for this purpose use Turk's solution (5 l leucocyte suspension + 495 µl of Turk's solution).
- Distribute the leukocytes in 96-well plates, 200 µl per well (300,000 cells/well).
- Incubate at 37°C with 5% CO2 for 3-4 days.

1.2.2 INOCULATION OF SAMPLES AND CONTROLS:

- After 3-4 days, dilute the erythrocytes 1/100 in 1X PBS and inoculate 20 µl of the dilution into each well.
- Perform a 1/10 dilution of each sample in the well to be tested (20 µl/well).
- Inoculate 8 wells per sample, leaving 8 free wells between samples to avoid contamination.
- Inoculate the positive control and negative control.
- Seal the plates with Parafilm.
- Incubate the 96-well plate at 37°C with 5% CO2 for 5-7 days to check for hemadsorption (HAD) or cytopathic effect (CPE).

1.3 ANALYSIS AND INTERPRETATION OF RESULTS:

- Inoculated wells should be observed daily under the microscope to check for the presence or absence of hemadsorptions (HAD) and/or cytopathic effect.
- The first observation can be made 14-16 hours after inoculation.
- To check for the presence of ADH, plates should be gently shaken before microscopic observation.
- The observation period should be extended to check for the presence of ADH or cytopathic effect up to 7 days after sample incubation.
- After 7 days, viral genome extraction and real-time PCR of the leukocyte culture supernatant from the plate is performed.
- Collection of 10 µl of the sample or biological matrix and a new pass is performed on a new plate with prepared leukocyte culture.

- This procedure is carried out a total of three times, thus obtaining three successive passes of the environmental sample or biological matrix on a leukocyte culture.

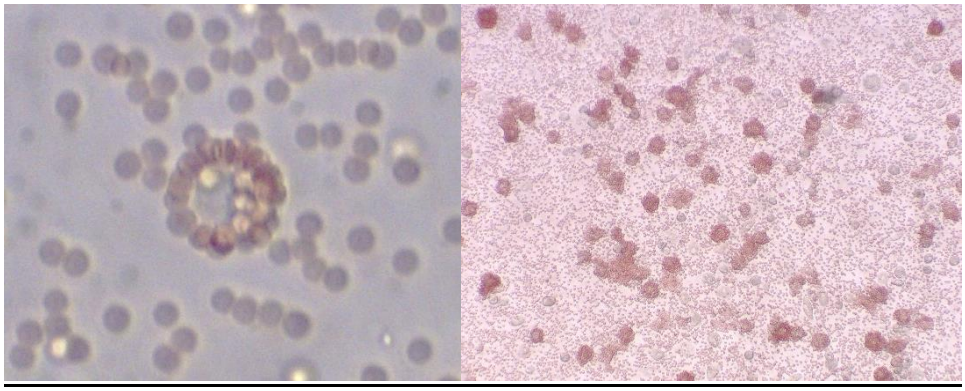

Figure 2. Hemadsorption.

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