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OIE Reference Laboratory

GENOTYPING OF AFRICAN SWINE FEVER VIRUS (ASFV) ISOLATES

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GENOTYPING OF AFRICAN SWINE FEVER VIRUS (ASFV) ISOLATES

*Equipment required

- Heating block or water bath
- Tubes shaker or vortex mixer
- Microcentrifugue for eppendorf tubes
- Convencional thermocycler with heated lid
- Tray for horizontal agarose gels, tank, combs, and powder leads
- UV transiluminator
- Microwave
- 4ºC fridge
- -20ºC or lower freezer

*General laboratory material

- Latex or nitrile gloves
- Permanent marker
- Tray with ice
- Bleach at 50%
- Racks for 10 ml tubes
- Racks for microcentrifuge tubes

1. CONVENTIONAL PCR

1.1 MATERIALS AND REAGENTS

- Micropipettes of volumes 1-20, 20-200, and 200-1000 µl
- Micropipette tips with aerosol resistant filter of 1-20, 20-200, and 200-1000 µl
- Microcentrifuge tubes of volumes 0.2 and 1.5 ml sterile
- Nuclease-free sterile H_2O, PCR grade.
- Platinum™ Green Hot Start PCR Master Mix (2x) (ThermoFisher Invitrogen™)
- Three different set of primers at a concentration of 10 pmol/ µl:
  - p72- U [5´- GGCACAAGTTCGGACATGT - 3´]
  - p72-D [5´- GTACTGTAACGCAGCACAG- 3´]

  C-terminal region of p72 protein
  - PPA89 [5´- TGTAATTTCATTGCGCCACAAC - 3´]
  - PPA722 [5´- CGAAGTGCATGTAATAAACGTC - 3´]

  Full *E183L*-gene encoding the p54 protein
  - CVR1 [5´- ACTTTGAAACAGGAAAC (AT) AATGATG -3´]
  - CVR2 [5´- ATATTTTGTAATATGTGGGCTGCTG- 3´]

  CVR within the *B602L* gen
- Positive ASFV DNA controls

For gel electrophoresis:

- Agarose (e.g. Biotools ref: 20.012)
- TAE 1XBuffer (e.g.Premixed TAE Buffer, 10X, Roche Diagnostics ref: 11 666 690 001)
- SYBR Safe (Invitrogen ref: S33102-400 µl) or ethidium bromide
- 10X loading buffer. For example:
  - Xinencianol (MERCK, ref:110590.0005)----------0.02gr
  - Azul de Bromofenol (PANREAC, ref: 251165.1604)-------0.02gr
  - Glicerol (PANREAC, ref: 131339.1211)----------3ml
  - Distilled water----------7ml
- DNA weight marker for amplicon size 257 bp (e.g. Biotools, ref:31.006 or Roche Diagnostics, ref: 0.019-1.11)

1.2 METHODOLOGY

A) DNA AMPLIFICATION

A.1 *MASTER MIX PREPARATION:*

- In a sterile 1.5 ml microcentrifuge tube, prepare the PCR reaction mix described below for the number of samples to be assayed (including positive and negative reaction controls). Make the calculations including for at least one extra sample due to the pipetting errors.
- Prepare three different PCR mix for one sample:

REAGENTS	***VOLUME*** (25 µl reaction)
H_2O	9.5 µl
Master Mix_Platinum	12.5 µl
Primer p72- U 10 µM	0.5 µl
primer p72- D 10 µM	0.5 µl

REAGENTS	***VOLUME*** (25 µl reaction)
H_2O	9.5 µl
Master Mix_Platinum	12.5 µl
Primer PPA89 10 µM	0.5 µl
primer PPA722 10 µM	0.5 µl

REAGENTS	***VOLUME*** (25 µl reaction)
H_2O	9.5 µl
Master Mix_Platinum	12.5 µl
Primer CVR1 10 µM	0.5 µl
primer CVR2 10 µM	0.5 µl

A.2 Add 23 µl of the PCR reaction mix to the required number of 0.2 ml PCR tubes.

A.3 Add 2 µl of DNA template (sample) to each PCR tube. Include a positive reaction control (2 µl of DNA) and a negative control (2 µl of distilled water) for each PCR run.

A.4 Close the reaction tubes and mix the content.

A.5 Place all tubes in an automated thermocycler equipped with heated lid. Run the **incubation program** detailed below:

Activation of polymerase	94ºC---2min	
DNA denaturation	94ºC---30sec	x 35 cycles
Primer annealing	55ºC---30sec	
DNA elongation	72ºC---1 min	
Extra elongation step	72ºC---5min.	
Hold at 4ºC		

B) AGAROSE GEL ELECTROPHORESIS:

B.1 Weight 2 gr of agarose to prepare a 2% agarose gel

B.2 Add 2 gr of agarose in 100 ml of TAE 1x Buffer

B.3 Heat the solution in the microwave until agarose is completely melted.

B.4 Add 10 µl of SYBR safe for each 100 µl of agarose. Shake carefully to disperse.

B.5 Prepare the gel tray, sealing the ends and placing the adequate number of combs. Pour the melted agarose into the gel tray. Wait until the gel become solid (aprox. 20 minutes).

B.6 Carefully remove the sealing of tray and place it into the tank. Remove carefully the combs. Add 1X TAE buffer to the tank until the gel is completely covered by TAE solution.

B.7 Add 2.5 µl of 10X loading buffer to each tube

B.8 Load 10 µl of each PCR sample to each well of the gel.

B.9 Add 10 µl of molecular DNA weight marker to one well one each lane of the gel.

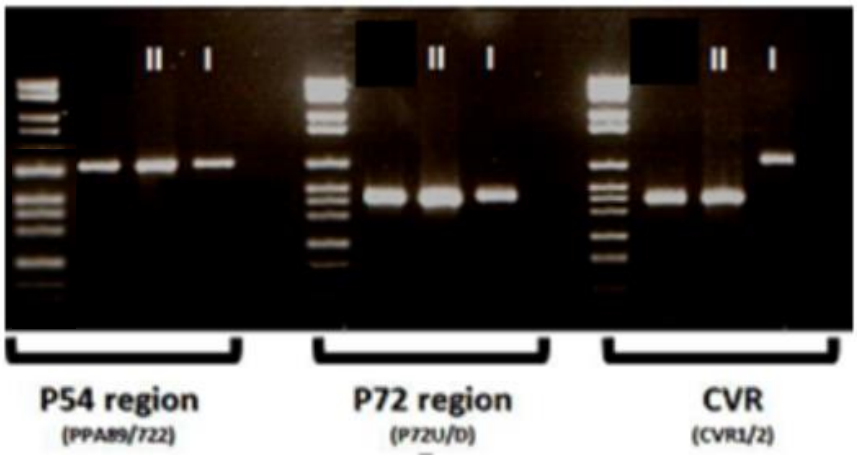
B.10 Connect the power supply. Run the gel at a constant voltage of 120-130 volts for about 30 minutes. (DNA samples have to move towards positive electrode)

B.11 For the lecture of the gel place it on an ultraviolet transilluminator.

1.3 RESULTS INTERPRETATION

A positive sample will present a discrete band that should have co-migrated with the PCR product of the Positive control. The molecular weight marker must be used as a reference to calculate the molecular weight of the product. The procedure will be valid if both extraction and reaction positive controls give a discrete band of the appropriate size corresponding to ASFV DNA amplicon, and both extraction and reaction negative controls do not give a band pattern.

The p72 U/D primers amplify a fragment of **~478 bp** within the C-terminal end of the p72 viral protein, which differentiates up to 24 distinct genotypes. The primers PPA89/722 amplify the whole sequence of the p54 viral protein giving raised amplicons of **~676 bp,** which is a valuable additional genotyping method for molecular epidemiological studies of p72 genotype I viruses, particularly in West Africa where this genotype predominates**.** The PCR amplification using primers CVR1/2 allow us to establish subtyping of the ASFV isolates based on size variations, e.g.: difference size is observed between genotype I and genotype II.



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