RESEARCH CENTER FOR ANIMAL HEALTH AND SAFETY (CISA-INIA)

SOP/CISA/ASF/VI /2/2008

(STANDAR PROCEDURE OPERATION FOR VIRUS ISOLATION IN MACROPHAGES FOR VIROLOGICAL DIAGNOSIS OF AFRICAN SWINE FEVER)

Rev. 1

Date: December 2008

REV.	DATE	EPIGRAPH	CAUSE OF CHANGE

Performed	Reviewed	Approved
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INDEX

1. PURPOSE

The main goal of this procedure is to describe the virus isolation technique to perform the African swine fever virus (ASFV) detection in porcine alveolar macrophages.

2. SCOPE

This procedure is applicable to porcine serum sample, blood with anticoagulant (EDTA) and porcine tissues. The target tissues for ASF are spleen, kidney, liver, lung, tonsil, heart, retro pharyngeal lymph node, renal lymph node, mesenteric lymph node and mediastinic lymph node.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

As a basic reference for the elaboration of this procedure it has been taken the criteria established in the next documents:

1. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). Capítulo 2.8.1. OIE, sexta edición, 2008. [http://www.oie.int/esp/normes/mmanual/A_00035.htm]
2. Ensayos de comparación interlaboratorial organizados por el Laboratorio Comunitario de Referencia.
3. PG/LCV/001 Procedimiento para la elaboración de documentos, Edición 01.
4. Borca M., Carrillo C, Zsak L, Laegreid WW, Kutish GF, Neilan JG, Burrage TG, Rock DL. (1998). "Deletion of a CD2-like gene, 8-DR, from African swine fever virus affects viral infection in domestic swine". J Virol Apr;72(4):2881-9.
5. Carnero R., Larenaudie, B., Ruiz-Gonzalvo, F. y Haag, J. (1967). Peste porcine africaine. Etudes sur la reaction d'hemadsorption et son inhibition par des anticorps specifiques. Rec. Vet. Med. 143, 49-59.
6. Carrasco L., de Lara FC, Martin de las Mulas J, Gomez-Villamandos JC, Hervas J, Wilkinson PJ, Sierra MA . (1996a). "Virus association with lymphocytes in acute African swine fever".Vet Res;27(3):305-12
7. Galindo I, Almazan F, Bustos MJ, Viñuela E, Carrascosa AL. (2000). "African swine fever virus EP153R open reading frame encodes a glycoprotein involved in the hemadsorption of infected cells". Virology Jan 20;266(2):340-51.
8. Malmquist, W. y Hay, D. (1960). "Hemadsorption and cytopathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures". Am. J. Vet. Res. 21, 104-108.

ASF REVIEWS:

1. Arias, M.; Sánchez-Vizcaíno, J.M. (2002). "African Swine Fever (ASF)". In Trends in Emerging Viral Infections of Swine. Iowa State University press, ISBN: 0813803837. Eds. A. Morilla, K-J Yoon, J. Zimmerman. Pp 119-124.
2. Arias, M.; Sánchez-Vizcaíno, J.M. (2002). "African Swine Fever Eradication: The Spanish model. In Trends in Emerging Viral Infections of Swine". Iowa State University press, ISBN: 0813803837. Eds. A. Morilla, K-J Yoon, J. Zimmerman. Pp 133-139.
3. Arias, M.; Sánchez, C.; González, M.A.; Carrasco, L. y Sánchez-Vizcaíno, J.M. (2002). "Peste porcina Africana" In curso digital de enfermedades infecciosas porcinas". [www.sanidadanimal.info] on line, July, 2002/.

3.2. DOCUMENTS TO BE USED TOGETHER WITH THIS PROCEDURE

- Procedure for antigen detection by conventional polymerase chain reaction (PCR) (SOP/CISA/ASF/PCR/1/2008)

4. BACKGROUND INFORMATION

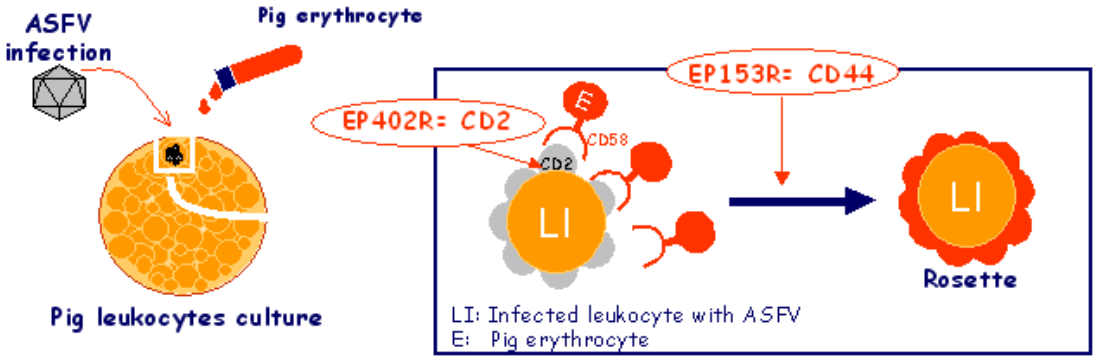
4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus
H.A.D; haemadsorption
C.E.P.; cytophatic effect
MAP; porcine alveolar macrophages
PC: Reference positive control
NC: Reference negative control
r.p.m: revolutions per minute

4.2. BACKGROUND

Malmquist and Hay made one of the most important advances in the study of African swine fever virus (ASFV) in 1960. They showed that ASFV was capable of infecting and replicating in primary leukocyte cultures from pig peripheral blood. When the virus replicates in such cultures, there is generally a **haemadsorption** reaction due to adsorption of pig red blood cells on ASFV infected leukocytes. Cell lysis follows after 48-49 hours of haemadsorption. The importance of this discovery relies on its specificity because none of the other pig viruses are capable of haemoadsorbing in leukocyte cultures.

The phenomenon of haemadsorption has been linked to two different genes of the ASFV genome. The ORF EP402R and ORF EP153R of Spanish isolate BA71. The first gene encodes a protein homologous to CD2, the cell adhesion receptor of T cells and an immune response modulator, and the second one encodes a protein homologous to CD44 molecules, involved in cellular adhesion and T-cell activation. In the case of the EP402R gene is responsible for the adhesion of swine erythrocytes to infected cells, and the EP153R is as a stabilizer of this adhesion.



Due to this specificity and from this time, it was possible to identify ASFV in vitro and the method could be used for diagnosis of the disease.

The haemadsorption test was used for first time in Spain in 1961. Since then, it has been used as a diagnostic method to control ASF and it has proved to be very valuable in the control of this epizootic disease. Other countries such as Portugal, Italy, Cuba, Brazil or Santo Domingo have also used haemadsorption test to diagnose and control this disease.

Today, the haemadsorption test is still the most sensitive technique to identify ASFV and is used for confirming the PCR positive results. However, it is laborious and slow in comparison with other methods employed in ASF diagnosis.

5. DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIAL

- Analytical Balance
- Centrifuge SORVALL RC6 (rotor SLA 1500)
- Chronometer
- CO_2 Incubator/ 37ºC
- Centrifuge tubes SORVALL 500 ml.
- Conic plastic tubes 12 ml
- Counter chamber THOMA or NEUBAHUER
- Distilled water
- Eppendorf tubes (or equivalent) 0,5ml; 1,5ml y 2ml.
- Filter MINISART 0,45 micras.
- Glass or plastic pipettes for volume of 1-25 ml
- Cryotubes
- Incubation bath
- Liquide Nitrogen Container
- Sterile funnel
- Laminar flow cabin class II
- Multichannel pipette 5-50µl
- Multichannel pipette 50-300 µl
- Nitrile gloves
- Phase Contrast Inverted cell Culture Microscope
- Pipetboy acu or equivalent
- Ph meter
- Reagent reservoir Polystyrene 50 ml [COSTAR Ref. 4870].
- Shaker plate
- Sterile disposable tips (1-10 µl, 1-200µ, 100-1000 µl).
- Sterile FALCON tubes 50 ml.
- Sterile glass bottle 250ml and 500 ml.
- Sterile forceps
- Sterile scalpel
- Sterile scissors
- Table centrifuge Megafuge1.0R (rotor Heraeus #7570)
- Tissue macerator

- Single channel pipettes 1-10 µl
- Single channel pipettes 10-100 µl
- Single channel pipettes 10-200µl
- Single channel pipettes 200-1000µl
- Disposable sterile tips
- 96 well cell culture plate's bottom flat (NUCLONTM "Surface", Nunc).
- Vortex.

REAGENTS

- **Citrate-dextrosa acid (ACD)**
  Trisodic citrate $2H_2O$ ---------- 11 gr
  Citric acid $1H_2O$ ---------- 4.34 gr
  Glucose ---------- 13.35 gr
  Distilled H_2O ---------- to 500 ml
  Sterilize by filtration and store at 4ºC

- **DMEM** [medium Dulbecco's Modified Eagle's con 4.5g/L Glucose -Bio-Whittaker]
  Store at 4º C
- **Freeze solution:** fetal bovine serum + 10 % DMSO (Dimetil sulfóxide)
- **Gentamicyne Sulfate** (50mg/ml) BioWhittaker.
  Store; 4º C
- **Glutamine** [4mM GIBCO]
  Aliquots 5ml/ Store -20º C
- **Erythrocytes lyses solution:** Ammonium Chloride 0,83% sterile (8,3 gr of NH_4Cl in 1L of distillate water)
  Store; 4º C
- **Fetal Bovine serum** [GIBCO]
  Aliquot 50ml/ Store -20º C
- **MAP Medium** DMEM [medio Dulbecco's Modified Eagle's with 4.5g/L Glucose -Bio-Whittaker] suplemented with 20% de SFB, 1% non esential aminoacids, 1% de glutamine, 1% sodic piruvate, 250µg/ml nystatin, and gentamicine.
- **Na Pyruvate** [Bio-Whittaker],
  Store; 4º C
- **Nystatine:** [10.000 U/ml, GIBCO]
- **Non essential aminoacids** [NEAA 100x BioWhittaker]
- **PC:** Reference positive control ASF virus. ASFV hemoadsorbent Spanish isolate E70
  Store; -70º C
- **Penicillin** [5.000U/ml)]**/Streptomicina** [5.000U/ml] – BioWhittaker
  Aliquots 5ml/ Store -20º C
- **Porcine alveolar macrophages (MAP)** obtained from naive porcine lung
- **Porcine erythrocytes** obtained from naive porcine peripheral blood
- **Porcine serum** obtained from naive porcine peripheral blood.
- **PBS buffer pH 7.2** .

ClNa [Merck 1.06404]	----------	8,0 g
ClK [Merck 1.04873]	----------	0,2 g
$P0_4H_2K$ [Merck 1.06586]	----------	0,2 g
$P0_4HNa_2$ [Merck 1.04936]	----------	1,15 g
H_20 distilled	----------	1000 ml

Check the pH before use. Store at room temperature.

- **Na Pyruvate** [Bio-Whittaker]
  Store; 4º C
- **Sulfato de gentamicina** [50mg/ml – BioWhittaker]
  Store at 4º C
- **Swine erythrocyte suspension diluted 1/10 PBS 1x**
- **Türk´s Solution:** commercial liquid with acetic acid and gentamina violet.
  Store at room temperature
- **Wash solution for lungs:** PBS1 x + gentamicine sulfate (50 mg/L) + 3% ACD
  Store at 4º C

5.2. PREPARATION

MACROPHAGES HARVESTING AND CULTURE.

Harvesting of alveolar macrophages from lungs.

The MAP are obtained from lung's piglets (2-3 weeks) perfunding the lungs through bronco-alveolar washing with a washing solution described in section 5.1 equipment and materials according to the following steps:

1. Close the trachea with a forceps before cutting.
2. Wash the lungs with distilled water removing the heart
3. Put the lungs into adsorbent paper and add with and sterile funnel 500-600 ml of washing solution.
4. Massage the lungs for 1-2 minutes and add the bronco-alveolar wash into an sterile bottle (this step is repeated twice)
5. Distribute the bronco-alveolar wash in centrifuges tubes (SORVALL) of 250 ml
6. Centrifuge at SORVALL RC6 centrifuge (rotor SLA 1500) 2.500g (4.000 r.p.m) for15 minutes at room temperature.
7. Resuspend the precipitate in 50 ml of washing solution and add to a sterile tube FALCON 50 ml
8. Centrifuge in Table Centrifuge [Megafuge 1.0R rotor Heraeus #7570mesa] 2.080 g (4.000 r.p.m) for 2 minutes at room temperature.
9. Repeat the washing step twice.
10. Remove the supernatant and resuspend the cell precipitate in a know volume (f.ex. 5ml) of SFB. Count the MAP in a counter chamber THOMA, and adjust the concentration to a final concentration of 5×10^7 cells (MAP)/ml
11. Distributed the resuspended cells in a frozen with a final concentration of 5×10^7 cells (MAP)/ml en in cryotubes (1ml/cyotube) and frozen in liquid nitrogen.

Culture of alveolar macrophages.

- Defrost one vial containing between 10^6 - 10^7 cells/ml.
- Wash the cells once with 5 ml of buffered salt solution (PBS 1x)
- Centrifuge the cell suspension for 10 minutes at 700g (room temperature) in table centrifuge
- Collect the cells in 10 ml of RPMI 1640 medium supplemented with 20% of FBS
- Dispense 100 µl of the cell suspension into each well of a microtitre plate at a concentration of 5x106 cells per ml.

The plates are incubated between 1-4 hours at 37º in CO2 atmosphere meanwhile the samples preparation.

Reference laboratory for ASF can supply MAP for performing the ASF virus isolation

SAMPLE PREPARATION:

Each sample must be identifying with a register number at CISA and with an identification number (sample ID) in case there are more than one sample from the same sender.

Serum sample (whole blood without anticoagulant);

1. If sample is blood without anticoagulant must be incubated 1 hour at 37ºC and after that, overnight at 4ºCfor the separation of the coagulum.
2. Discard the coagulum and centrifuge in a table centrifuge [Megafuge 1.0R rotor Heraeus #7570] at 780g (1.500 r.p.m) during 10 minutes.
3. Recover the supernatant and put in a MINISART filter of 0,45 micras.
4. Add 1% of gentamicyne sulphate to the filtered supernatant and keep 1 hour at 4º.
5. The treated sample is put in an eppendorf tube, which its register entry numbers CISA and the ID of the sample.

Whole blood sample with anticoagulant;

1. Prepare a 1/10 dilution of whole blood in sterile PBS1x pH 7,2
2. Add 1% of gentamicyne sulphate to the filtered supernatant and keep 1 hour at 4º.
3. The treated sample is put in an eppendorf tube, which its register entry numbers CISA and the ID of the sample.

Tissues;

1. After the organ is ground in a tissue homogenize, prepare a cell suspension at 10% with sterile PBS1x pH 7,2. (1g tissue/ 10 ml sterile PBS1x).
2. Centrifuge in a table centrifuge [Megafuge 1.0R rotor Heraeus #7570] at 1.050g (2.000 r.p.m) during 10 minutes.
3. Recover the supernatant and put in a MINISART filter of 0,45 micras.
4. Add 1% of gentamicyne sulphate to the filtered supernatant and keep 1 hour at 4º.
5. The treated sample is put in an eppendorf tube, which its register entry numbers CISA and the ID of the sample.

5.3. METHODS

1. After 1-4 hours inoculated the MAP with 1/10 dilution (10 µl/well) of treated sterile sample. If it is possible inoculated at least four wells per sample.

2. Leave four wells as positive control (PC) and four wells as cell control (negative control).Uninoculated negative controls are essential to monitor the possibility of nonspecific haemadsorption.

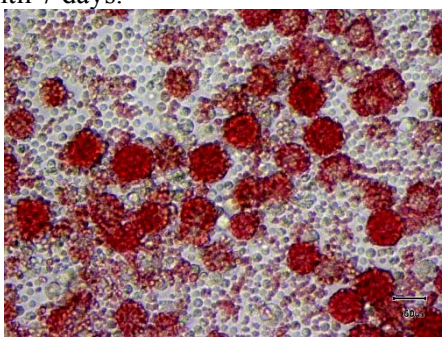
3. Add 20 µl per well of a fresh preparation of 1% pig erythrocytes in buffered salt solution to each tube (final dilution 1/100 in PBS 1x sterile).

4. Incubate at 37º in CO_2 incubator. Read the plates every day for 7 days to check the presence of HAD or c.p.e.

5.4. RESULTS

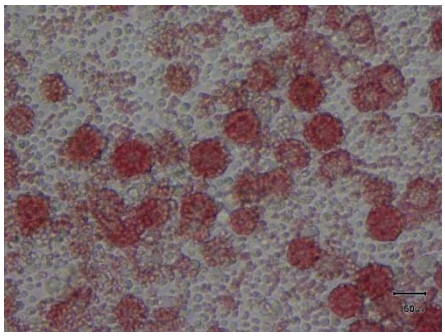
NOTE: At the moment of reading results, each well is analyzed as individual well comparing with the pattern observed in the PC and the results obtained in the wells without inoculated. In this way, samples will be analyzed respect to the controls of its plate.

Reading the results.
The inoculated well will be read every day in the microscope to check the presence of **a positive HAD and/or CPE.** The first read can be performed at 14-16 hours post inoculation.
To check the presence of the HAD positive results in the microscope the plates must be gentile shaken allowing the read of the HAD positive results. The read period must be extended until check the presence of HAD and/or CPE positive results until 7 days.



Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells (rosette) . A CPE consisting of a reduction in the number of adherent cells in the absence of haemadsorption may be due to the cytotoxicity of the inoculum, Aujeszky's disease virus or non-haemadsorbing ASFV, which can be detected by the FAT on the cell sediment or by use of PCR. If no change is observed, or if the results of the immunofluorescence and PCR tests are negative, subinoculate the supernatant into fresh leukocyte cultures.

CPE+/PCR +/ HA - → Nonhaemadsorbing ASFV
CPE+/PCR -/ HA - → Cytotoxic (no ASFV)



5.5. CRITICAL POINTS

In the last years has been analyzed a lot of sample by VI, with good results of specifity and sensitivity for virological diagnosis of ASF. But there are some critical points:

1. **The procedure is long and laborius.** To give a correct ASF diagnosis it is required between 5-10 daysy the HAD pattern if observed in the first passage. If not can be delay until 15-30 days. It is not choose as election technique to perform the virological diagnosis of ASF. It is used as confirmatory technique for PCR positive results.

2. The presence of CPE without HAD can be due to the presence of additional viruses or to a cytotoxic effect. This issue makes more difficult a correct diagnosis of the disease and requires a late PCR confirmation.

3. Previous studies have demonstrated that **badly conserved sample could origin false reaction avoiding the isolation of the ASFV.** The samples must be kept in a cold chain during the storage and transport.

4. Previous studies have demonstrated the **influence** of lyophilized samples **in the HAD technique decreasing the** effectiveness

5. This technique requires a Laboratory with cell culture conditions and animal facilities department.

5.6. SAFETY CAUTIONS

- Read the protocol previously.
- Work in sterile conditions to avoid the cell culture contamination.
- Avoid any reagent contamination
- Do not eat, smoke or drink while the manipulation of reagents.
- Do not pipette by mouth.
- Use a new tip for each sample.
- Always include PC, and NC.

6. APPENDIX

Appendix 1. FORM CISA/PPA/VI/2/2008

ENTRY REGISTER CISA:
CELLS:
DATE CELL CULTURE:
DATE CELL INOCULATION:
TECHINICIAN:

	1	2	3	4	5	6	7	8	9	10	11	12
A												PC
B												PC
C												PC
D												PC
E												NC
F												NC
G												NC
H												NC

OBSERVATIONS: