

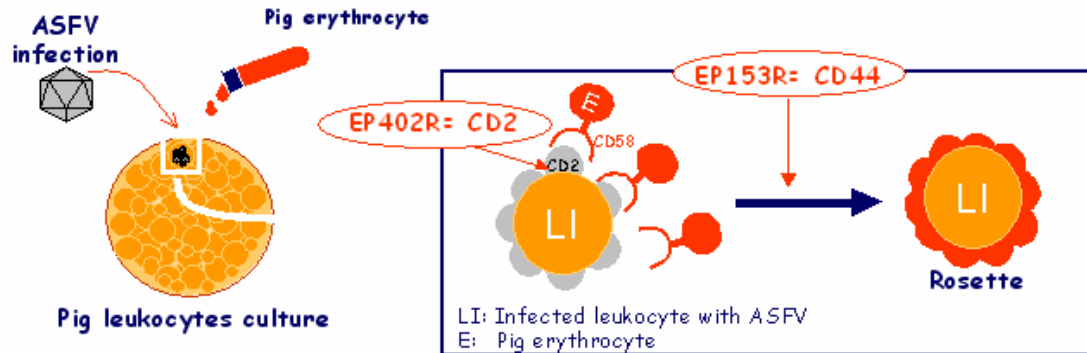


AFRICAN SWINE FEVER VIRUS DETECTION: HAEMADSORPTION TEST IN PRIMARY LEUCOCYTE CULTURES

- **INTRODUCTION**

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 haemadsorbing 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. However, it is laborious and slow in comparison with other methods employed in ASF diagnosis.

• MATERIALS AND REAGENTS REQUIRED

Cells:

- **LEUKOCYTES:** obtained from defibrinated swine blood
- **MAP:** porcine alveolar macrophages from donor pig.

Culture Mediums and plastic material:

- **Swine leukocyte culture;** the leukocytes are cultured in homologous porcine serum from the same donor pig.



- **MAP are cultured in EMEM** (minimal essential medium EAGLE -Bio-Whittaker-); supplemented with 20% of FBS (Bio-Whittaker), 1% essential amino acids, 1% glutamine (4mM) (GIBCO) and gentamicine (50mg/ml) de Bio-Whittaker.
- 96 micro titter well plates (NUCLON™ “Surface”, Nunc).
- THOMA or NEUBAUER counter chamber for counting the total leucocytes

Buffers required:

- Lung washing solution; EDTA 2 mM in PBS pH 7.4, Penicillin 500 U/ ml, Gentamicine 40 µg/ml y Glucose 1 mg/ml.
- MAPs washing solution; RPMI-M α medium with FBS 11,5%, Hepes 20 mM pH 7, Penicillin 400 U/ ml, Gentamicine 56 ug/ml, L-Glutamine 2.4 mM y 2-Mercaptoetanol 0,05 mM.
- Frozen solution; SFB + DMSO 10%
- Erythrocytes lyses solution: ammonium chloride 0,83% .

NH₄Cl ----- 8,3 gr.

H₂O ----- 1000 ml.

- PBS buffer pH 7.2

NaCl (Merck 1.06404)	8.0 g
KH ₂ PO ₄ (Merck 1.04873)	0.2 g
Na ₂ HPO ₄ 12 H ₂ O (Merck 1.06586)	2.9 g
KCl (Merck 1.04936)	0.2 g
Distilled water to	1000 ml

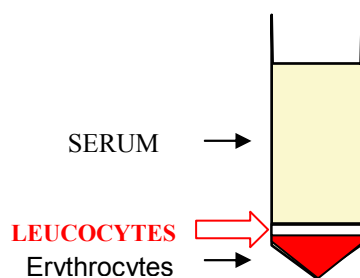
Check the pH before use. Store at 4°C.

- Turk colorant; commercial reagent.

• METHODS

1. Leucocytes harvesting and culture.

1. Collect the required volume of fresh pig blood defibrinate. The blood is collected by puncture in cava vein to defibrinate and is shaking during 15-30 minutes.
2. The blood defibrinate dispense in aliquots of 50 ml (falcon tubes) or centrifuge tubes of 500 ml and centrifuge at 700g rpm for 30 minutes without brake.
3. The blood is separated in three fractions, the sera (culture medium for leucocytes), a fine white layer (leucocytes) and the third fractions are the erythrocytes. The three fractions are collected:



- **The serum** is collected in a bottle of 500 ml for being used as culture medium of the leukocytes.
- **The fine white layer** (leucocytes) is collected by capillarity avoiding collecting the red cells (erythrocytes).
- Collect **the erythrocytes** diluted 1/10 in PBS 1x. Culture pig leukocytes in a micro titre plate. Separate leukocytes from blood by a conventional method.

NOTE: for the specificity of the test is necessary to use the serum and the erythrocytes of the pig from which the leucocytes has been obtained (homologous serum). This fact avoids unspecific agglutinations reactions.

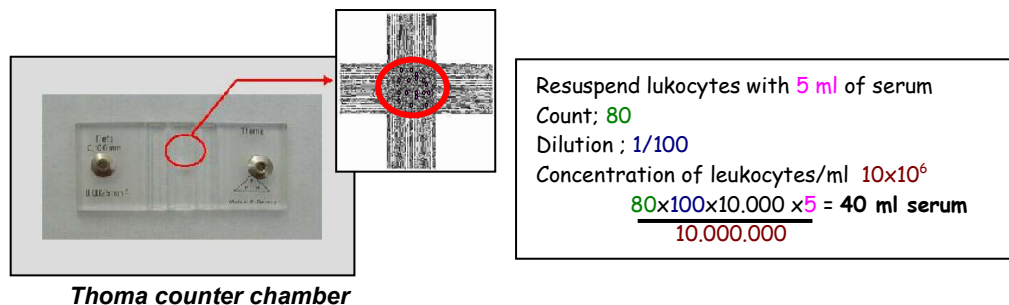
4. Add three volumes of 0,83% of ammonium chloride to the leukocytes obtained. Mix and incubate at room temperature for 15 minutes.
The ammonium chloride allows the lyses of the erythrocytes remained.
5. Centrifuge at 650g (15 min).



- Carefully remove the supernatant and add three volumes of 0.83% ammonium chloride. Mix and incubate at room temperature for 15 minutes.
- Centrifuge at 650g (15 min).
- Carefully remove the supernatant and collect the pellet (clean leucocytes) with 5-10ml of homologous serum collected in the third step.
- Immediately count the leukocytes and adjust the concentration of the suspension to a final concentration of $8-10 \times 10^6$ leukocytes per ml of serum.

Example:

- 5 μ l of leukocytes resuspend with 5 ml of homologous porcine serum + 495 μ l of Turk colorant.
- Vortex
- 25-30 μ l are put in the THOMA or NEUBAUER counter chamber



Thoma counter chamber

- Finally, distribute the leukocytes culture in 96 micro titer well plates adding 200 μ l per well (300,000 cells/ per well) and incubate at 37 °C in CO₂ for 3-4 days allowing the maturation of the leukocytes.

2. Porcine alveolar macrophages (MAP) harvesting and culture.

Harvesting of alveolar macrophages from lungs.

The macrophages should be harvested from the lung on the same day that the pig is slaughtered. The lungs should be washed three or four times with water and a total volume of approximately 200 ml sterile phosphate buffered saline (PBS). The harvested wash fluid is then centrifuged for 15 minutes at 700g. The resulting pellet of macrophages is resuspended in 30 ml of *lung washing solution* and centrifuged (washed) twice more. The final pellet is

resuspended in *frozen solution* and the number of macrophages is counted to determine the cell concentration. The macrophages can then be used fresh, or can be stored in liquid nitrogen according to standard procedures at a final concentration of approximately 5×10^7 macrophages/ml. Macrophage batches should not be mixed.

Culture of alveolar macrophages.

Defrost one vial containing 5×10^7 macrophages/ml. Wash the cells once with 5 ml PBS and centrifuge the cell suspension for 10 minutes at 700g (room temperature) in table centrifuge. Collect the cells in 10 ml **EMEM** (minimal essential medium EAGLE -Bio-Whittaker-) supplemented with 20% of FBS. Dispense 100 μ l of the cell suspension into each well of a microtitre plate at a concentration of 5×10^6 cells per ml.

The plates are incubated for 4 hours at 37° in CO₂ atmosphere.

3. Virus isolation on leucocytes and on alveolar macrophages

Preparation of sample (serum, blood, 10% tissue suspension).

- After the organ is ground in a tissue homogenize, prepare a cell suspension at 10% with PBS solution.

- Centrifuge at 600g for 20 minutes

Take the supernatant and add antibiotics (4,000 U.I. penicillin and 3mg streptomycin per ml). Incubate for 2 hours at room temperature or 1 hour at 4°.

The sample (tissue, blood or serum) can be also filtrated with a filter of 45 micron of diameter.

Incubation of samples

- Inoculate the leucocytes (after 3-4 days) or the MAP (after 4 hours) with 20 μ l of samples (4 wells per sample at least).
- Inoculate positive control cultures with haemadsorbing virus. Uninoculated negative controls are essential to monitor the possibility of nonspecific haemadsorption.
- Incubate 24 hours at 37° in CO₂ atmosphere

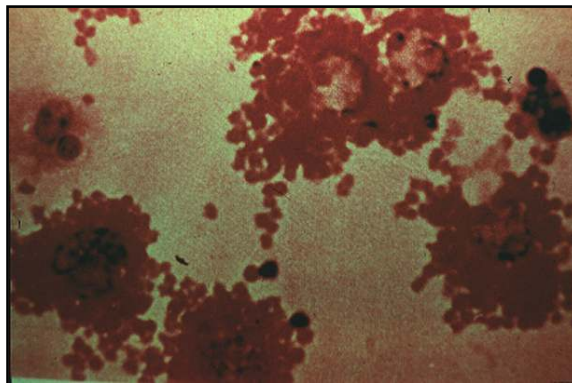
4. Haemadsorption test.

After 24 hours, add 20 μ l pre well of a fresh preparation of 1% pig erythrocytes in buffered saline to each tube. **It is very important to use erythrocytes from the same animal as the leukocytes because this avoids non-specific agglutination reactions.**

- **READING AND INTERPRETING THE RESULTS.**

Examine the cultures daily for 7-10 days under a microscope for cytopathic effect (CPE) and haemadsorption.

Reading the results: Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells. 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 nonhaemadsorbing 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.



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