

# The role of diagnosis in the control of ASF



***TAFS forum. Scientific Conference on  
ASF, Beijing, 2019***

**Prof. JM. Sánchez-Vizcaíno**

**Complutense University of Madrid  
Visavet Center**

***OIE-ASF Reference Laboratory***

***[www.sanidadanimal.info](http://www.sanidadanimal.info)***

***[jmvizcaino@ucm.es](mailto:jmvizcaino@ucm.es)***

Laboratory diagnosis is  
essential in the ASF control

**Different clinical presentation**

Common lesions with other H. diseases

**A FAST RESPONSE IS  
NEED**

# ASF LABORATORY DIAGNOSIS

## KEY POINTS

• **No Vaccine Available** → **Antibodies = INFECTION**

- **No Neutralizing Antibodies**

ASFV-specific antibodies do not neutralise virus in the classical concept of neutralization- only a partial neutralization “in vitro” has been demonstrated.



- **Viremia for Long period of Time**

- **Antibodies Persist During Month , Even Years**

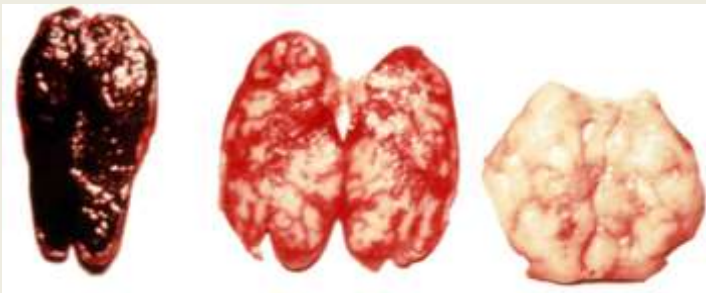
From 7-12 dpi.

**Abs** good infectious marker

- **Antigen – Antibody Immunocomplex Formation**

Low sensitivity in the direct antigen detection techniques

# ITS ROLE IN THE EARLY DETECTION

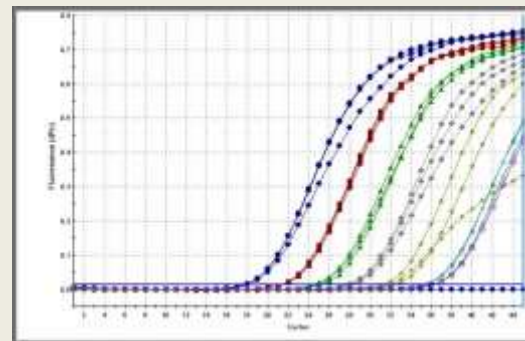


## FIELD:

- Risk information
- ASF Information
- Sample to Laboratory

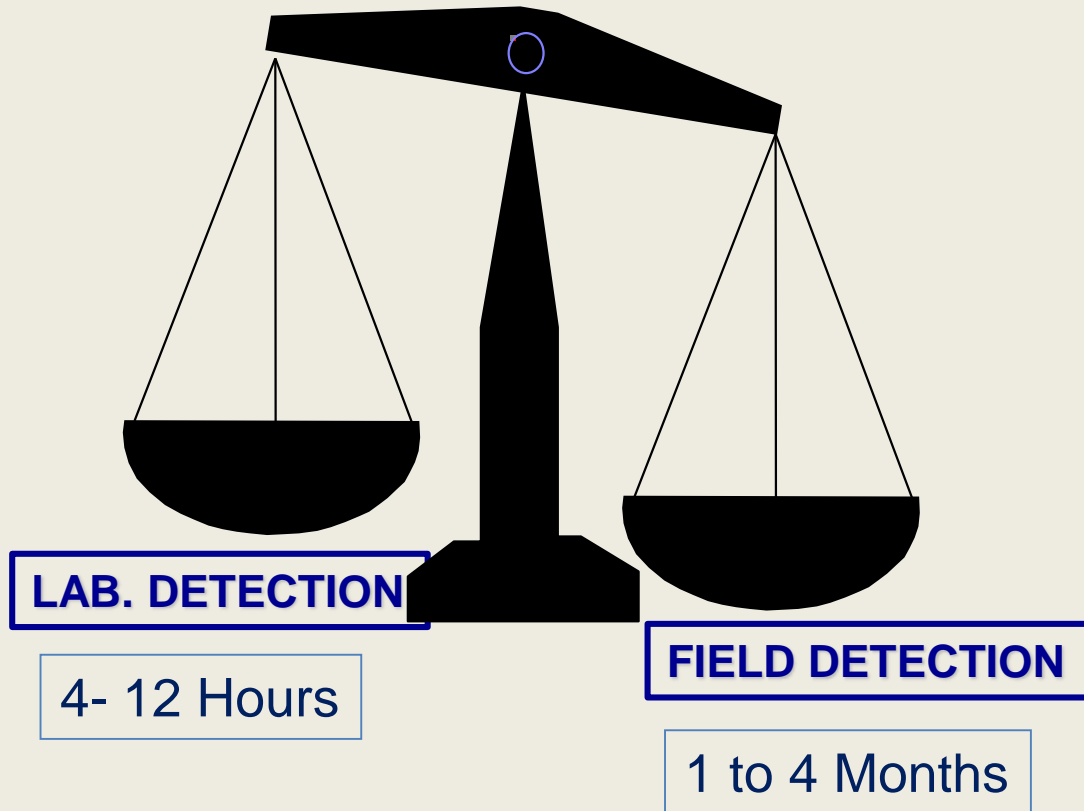
## LABs:

- Good connection with field
- Good test and procedure



**TRAINING: FIELD AND LABORATORY**

# LACK OF EQUILIBRIUM



# ASF FIELD: CLINICAL SIGNS

## Different clinical forms

### PERACUTE-ACUTE-SUBACUTE

- Virulent isolates
- 80-95% mortality
- Similar to other porcine haemorrhagic diseases
- No pathognomonic lesions

### SUBCLINICAL-ASYMPTOMATIC

- Areas where ASF is endemic
  - Less virulent isolates
- Moderate-Low mortality
  - Inapparent carriers

# Acuate-subacute forms of ASF:

*Could be Easily Confused WITH:*

Fever

- Erysipelas
- Salmonellosis
- Actinobacillus (App)
- Other Septicaemic conditions
- PDNS

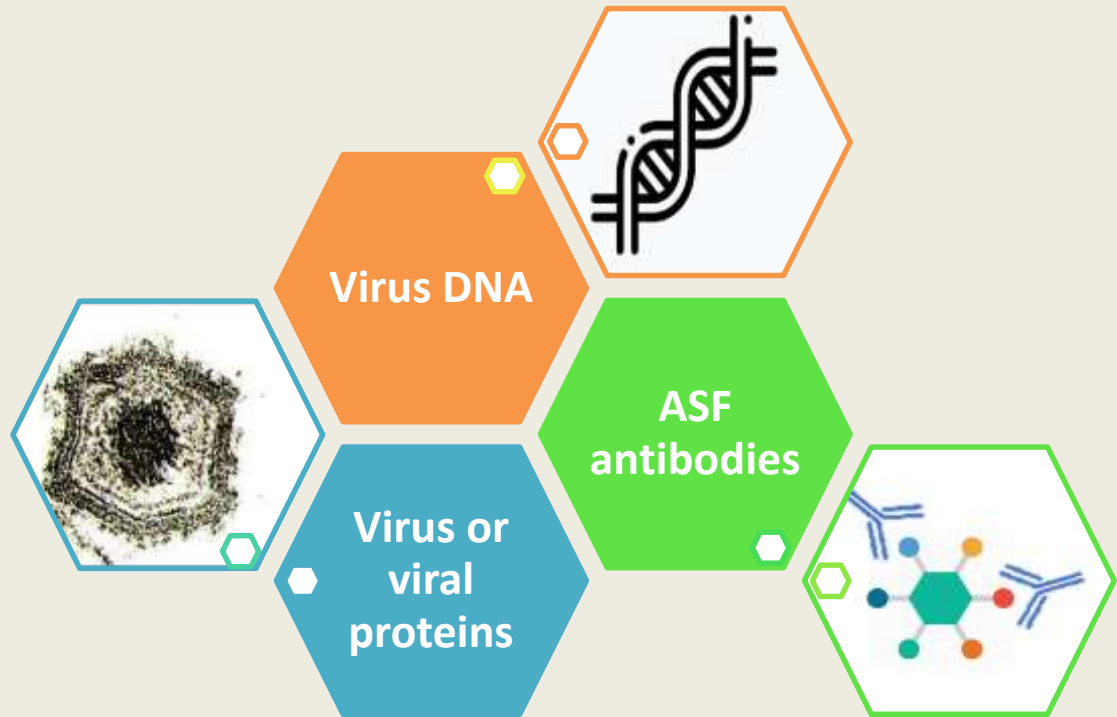


**Thereby laboratory diagnosis is essential**

# ASF LABORATORY DIAGNOSIS

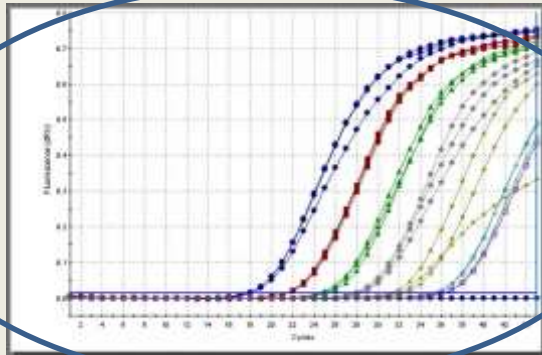
IS in GOOD FORM for the 24 Genotypes

**P72**  
**pp62**  
**P32 (30)**  
**P54**  
**p35-23.5**  
**P12**

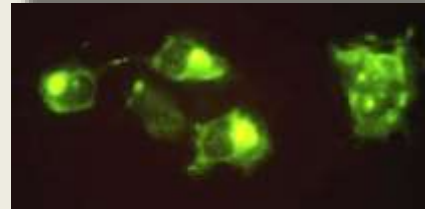
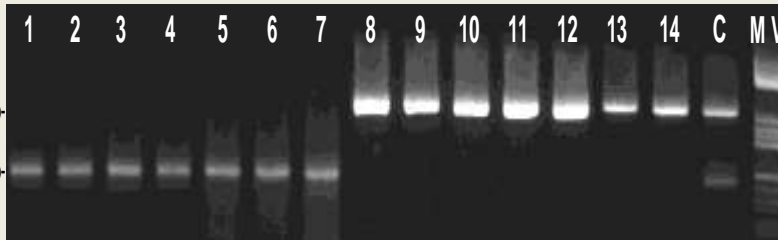


**ALWAYS: AB + VIRUS DETECTION IN PARALLEL**

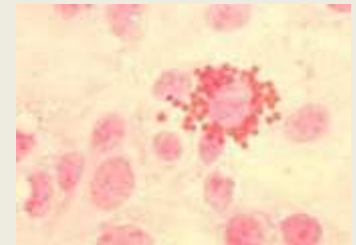
# ASF LABORATORY DIAGNOSIS



**GOOD HEALTH**



*CSF-3/4 + ASF-1/2*



**Monitoring the detection of both: Antigen-DNA and Antibodies it's critical for ASF control. Can be not excluded**

# Samples



- Blood and serum



- Lymph nodes



- Spleen



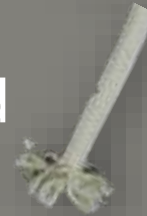
- Lungs



- Kidney



- Oral fluid



- Faeces

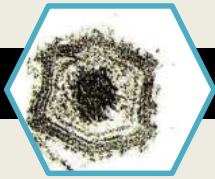


# Samples

- Blood and serum
- Lymph nodes
- Spleen
- Lungs
- Kidney
- Oral fluid
- Faeces



**NO  
BLOOD!!!**



# IDENTIFICATION OF ASFV

VIRUS ISOLATION +  
HAEMADSORPTION  
(HA) TEST

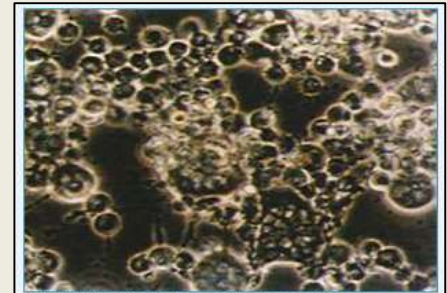
Reference test to confirm  
ASF presence

Malmquist and Hay, 1960

Samples (+ASFV)

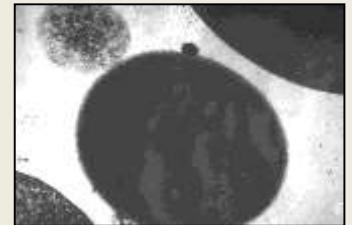


Susceptible  
primary culture  
(monocytes and  
macrophages)



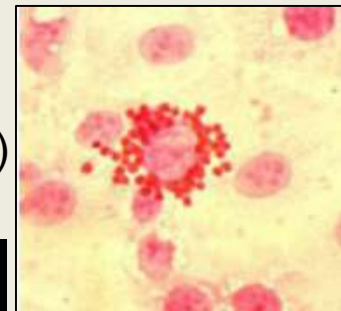
*Cytopathic effect of a porcine macrophage culture infected with ASFV.*

Cytopathic effect (CPE) → daily observation 7-10  
days → CPE after 48 hours HAD



**Positive HAD** → always **ASF POSITIVE**  
**NO POSITIVE HAD** → COULD BE ASF POSITIVE (PCR)

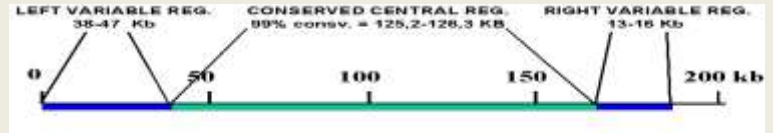
**Some ASFV No HA. MAINLY ATTENUATED ISOLATES**





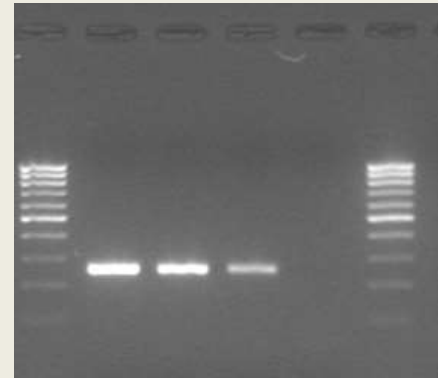
# IDENTIFICATION OF ASFV

## PCR CONVENTIONAL and PCR REAL TIME



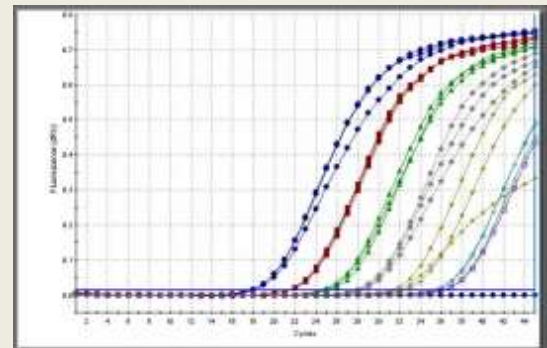
### Conventional PCR [Agüerro et al., 2003, 2004]

- VP72 (73) protein (88,363-88,619 nt) → viral capsid  
 F → 5'-AGT-TAT-GGG-AAA-CCC-GAC-CC-3'  
 R → 5'-CCC-TGA-ATC-GGA-GCA-TCC-T-3'
- PCR product → 257 base pairs



### Real time PCR [King et al., 2003]

- VP72 (73) protein (2041-2290 vp72 gene seq) → viral capsid
- Taqman probe  
 F → 5'-CTGCTCATGGTATCAATCTTATCGA-  
 R → 5'-GATACCACAAGATCRGCCGT-3'
- PCR product → 250 base pairs





# New techniques for ASF diagnosis

## UPL REAL TIME PCR

### Currently used

Fernández-Pinero et al., 2013

### Protocol described in Fernández-Pinero, 2013

### The highest sensitivity

survivor animals

early stages of infection

Based on **vp72** encoding region

**UPL probe** (ProbeFinder Software)

Specific primers

PCR target	Name	Sequence (5'-3')	Nucleotide position	ASFV strains homology (%) <sup>a</sup>
ASFV UPL PCR	ASF-VP72-F primer	CCCAGGCGATAAAATGACTG	893-912 <sup>b</sup>	100
	ASF-VP72-R primer	CACTAGTTCCTCCACCGATA	940-960 <sup>b</sup>	100
	UPL#162 probe	6FAM-GGCCAGGA-dark quencher dye (Roche cat no. 04694490001)	930-937 <sup>b</sup>	100
$\beta$ -actin UPL PCR	ACT-162-F primer	GGATGCAGAAGGAGATCACG	1022-1041 <sup>c</sup>	NA
	ACT-162-R primer	ATCTGCTGGAAGGTGGACAG	1132-1151 <sup>c</sup>	NA
	UPL#162 probe	6FAM-GGCCAGGA-dark quencher dye (Roche cat no. 04694490001)	1121-1128 <sup>c</sup>	NA

NA, not applicable; UPL, Universal Probe Library.

<sup>a</sup>Nucleotide homology for the 38 ASFV sequences aligned with ClustalW 2.0.

<sup>b</sup>ASFV Spain 70 VP72 gene (GenBank accession no. 589966).

<sup>c</sup>Sus Scrofa  $\beta$ -actin gene (GenBank accession no. AY550069).

**Positive controls → 30-36 Ct**

**Negative controls ≥ 40 CT**

**Ct > 38 doubtful**

# Comparison of techniques targeting antigen detection

Comparative viremia results determined by the **OIE real-time PCR (red)** and **UPL real-time PCR (blue)** in blood samples collected from exposed (A) and inoculated (B) pigs using the ASFV genotype II Lithuania 2014 isolate

**TABLE 4** Comparison of UPL real-time PCR, OIE real-time PCR, OIE conventional PCR, and antigen ELISA results for the detection of ASFV in blood and tissues collected from pigs experimentally infected with genotype II ASFV isolates

			UPL-PCR		OIE real-time PCR		OIE conventional PCR		Ag-ELISA (Ingenasa)	
ASFV strain by sample type	No. of pigs examined	No. of samples examined	No. of positive samples	%	No. of positive samples	%	No. of positive samples	%	No. of positive samples/total no. of animals	%
Blood samples										
Ukr12/Zapo	6	19	10	52.6	10	52.6	10	52.6	9	47.3
Arm07	6	20	10	50	10	50	10	50	10	50
LT14/1490	18	111	42	37.83	39	35.13	32	29.8	29	26.12
Total	30	150	62	41.3	59	39.3	52	34.7	48	32.0
Tissue samples										
Ukr12/Zapo	6	90	90	100	90	100	90	100	6/6 <sup>a</sup>	100
Arm07	6	90	90	100	90	100	90	100	6/6 <sup>a</sup>	100
LT14/1490	18	270	260	96.29	255	94.4	255	94.4	16/18 <sup>a</sup>	88.8
Total	30	450	440	97.8	435	96.6	435	96.6	28	93.3

<sup>a</sup> In Ag-ELISA, spleen samples for each pig were included in the study.



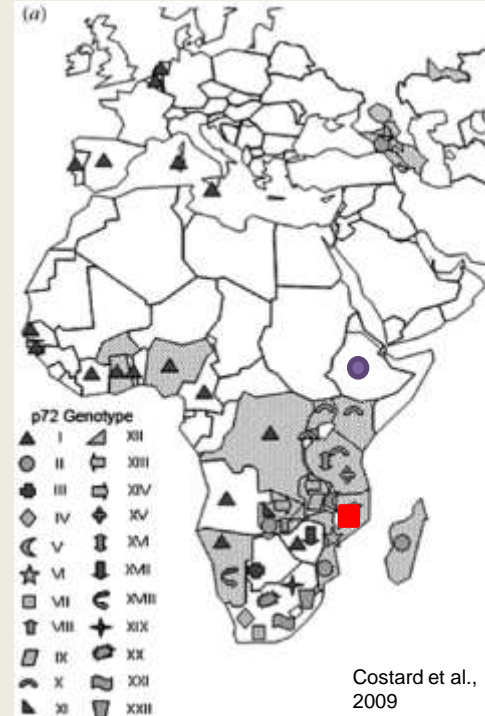
# Genotyping strategy

Distinguishing between **genotypes** → C-terminal end of the gene B646L encoding the major protein p72

So far, **24 genotypes** have been identified

Additional genotyping method Genotype I → sequence the full E183L-gene encoding the p54 protein → **subtypes within Genotype I**

Distinguishing between **closely related isolates**



Costard et al.,  
2009

Transboundary and Emerging Diseases

Original Article | Open Access | CC BY

### Identification of a New Genotype of African Swine Fever Virus in Domestic Pigs from Ethiopia

J. E. Adenbach, C. Gallardo, E. Nieto-Pelegrin, B. Rivera-Arroyo, T. Degefa-Negi, M. Arias, S. Jenberie, D. D. Mulisa, D. Gizaw, E. Gelaye, T. R. Chibssa, A. Belaye, A. Loitsch, M. Forsa, M. Yami, A. Diallo, A. Soler, C. E. Lamien, J. M. Sánchez-Vizcaino. ... See fewer authors

First published: 22 May 2016 | <https://doi.org/10.1111/tbed.12511> | Cited by: 12

Transboundary and Emerging Diseases

ORIGINAL ARTICLE | Open Access | CC BY

### Genetic characterization of African swine fever virus isolates from soft ticks at the wildlife/domestic interface in Mozambique and identification of a novel genotype

C. J. Quembo, F. Jori, W. Vosloo, L. Heath

First published: 17 September 2017 | <https://doi.org/10.1111/tbed.12700> | Cited by: 2



# IDENTIFICATION OF ANTIBODIES AGAINST ASFV

**OIE ELISA**

**IMMUNOBLOTTING**

**INDIRECT FLUORESCENT  
ANTIBODY TEST**

**COMMERCIAL ELISA**

198: Version adopted by the World Assembly of Delegates of the OIE in May 2012

**SECTION 2.8.**

**SUIDAE**

**CHAPTER 2.8.1.**

**AFRICAN SWINE FEVER**



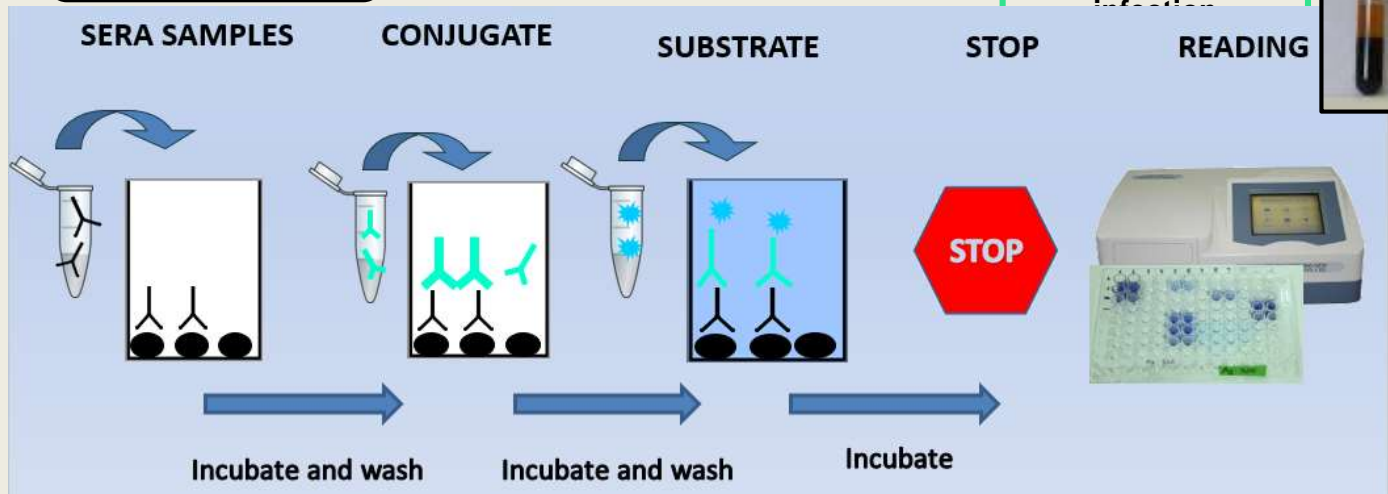
# IDENTIFICATION OF ANTIBODIES AGAINST ASFV

## OIE ELISA

Fast and low cost → screening

Sánchez-Vizcaíno et al., 1979, 1982, Pastor et al., 1989;

Antibodies always  
synonym of  
infection



Cut off calculation

$CUT\ OFF = Optical\ Density\ negative\ serum \times 1 + Optical\ Density\ Positive\ serum \times 0.2$

- Sera with an optical density below the CUT OFF - 0.1 can be considered negative.
- Sera with an optical density higher than CUT OFF + 0.1 can be considered positive.
- Sera with an optical density between CUT OFF ± 0.1 can be considered inconclusive and the result needs to be confirmed by the IB technique.



# IDENTIFICATION OF ANTIBODIES AGAINST ASFV

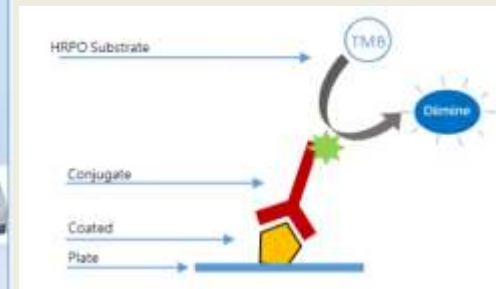
## COMMERCIAL ELISA

**INGENASA PPA COMPACT** (blocking ELISA)

Antigen vp72 (73)



Antibodies always  
synonym of  
infection



<sup>6</sup>Commercial ELISA tests for antibody detection: INGEZIM PPA COMPAC K3 (INGENASA); ID Screen, ID-VET; SVANOVIR ASFV-Ab; SVANOVIR



# IDENTIFICATION OF ANTIBODIES AGAINST ASFV

## IMMUNOBLOTTING

### Confirmatory test

Escribano et al., 1990; Pastor et al., 1989

Antibodies always  
synonym of  
infection



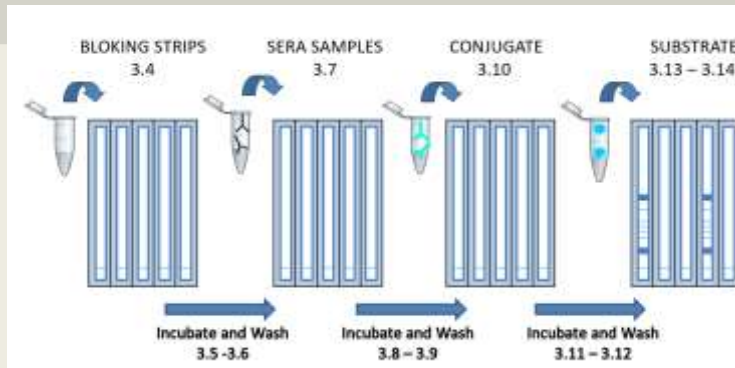
**High specificity**, more objective and easier interpretation than indirect immunofluorescence

Better **recognition of weak positive samples**

Same antigen than in OIE ELISA test → ASFV polypeptides are placed on strips

Useful when  
samples have been  
incorrectly handled  
or preserved

**POSITIVE  
SAMPLES WILL  
REACT WITH  
SEVERAL  
PROTEINS**



EU reference lab web



# Comparison of techniques targeting antigen detection

Comparative viremia results determined by the **OIE real-time PCR (red)** and **UPL real-time PCR (blue)** in blood samples collected from exposed (A) and inoculated (B) pigs using the ASFV genotype II Lithuania 2014 isolate

Sample type	OIE real-time PCR		OIE conventional PCR		Ag-ELISA Ingenasa	
	No. of positive samples/total no.	Ss (% [95% CI]) <sup>a</sup>	No. of positive samples/total no.	Ss (% [95% CI])	No. of positive samples/total no.	Ss (% [95% CI])
Experimental	494/502	98.4	487/502	97.0	76/92	82.6
Field	291/295	98.6	284/295	96.3	66/92	71.7
Total	785/797	98.5 (97.4–99.1)	771/797	96.7 (95.3–97.8)	142/184	77.2 (70.6–82.6)

<sup>a</sup> Ss, sensitivity.

**UPL USED  
AS  
REFERENCE  
TEST**

Gallardo et al., 2015



# New techniques for ASF diagnosis

## IMMUNOPEROXIDASE TEST

**Currently used**  
**Alternative confirmatory**  
**serological test**

**Antibodies always**  
**synonym of infection**

ASFV infected Vero and MS cells

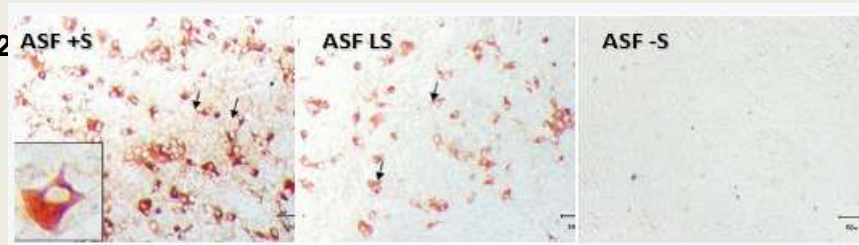
Virions migrates to the membrane cell from which it gets a new envelope

Immune-cytochemistry technique → antigen-antibody formation (peroxidase enzyme)

SOP at <http://asf-referencelab.info/asf/en/procedures-diagnosis/diagnostic-procedures>

## Technique fully validated at the EURL for ASF

**Sensitivity 98.2**  
**and IFI)**



EU reference lab web

# Comparison of techniques targeting antibody detection

Comparative **IPT** and **ELISA** results obtained in serum samples from seroconverted animals experimentally infected with genotype II ASFV isolates

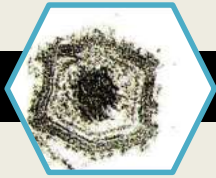
ASFV isolate	Animal identification	dpi/dpe <sup>a</sup>	Result for <sup>b</sup> :				
			IPT	OIE-ELISA	Ingenasa-ELISA	IDvet-ELISA	Svanova-ELISA
Arm07	Contact pig 5	16	Pos	Neg	Pos	Neg	Neg
LT14/1490	Inoculated pig 6	18	Pos	Neg	Neg	Neg	Neg
LT14/1490	Contact pig 2	21	Pos	Neg	Neg	Neg	Neg
LT14/1490	Contact pig 10	17	Pos	Neg	Neg	Neg	Neg
LT14/1490	Contact pig 11	17	Pos	Neg	Neg	Neg	Neg
LT14/1490	Contact pig 11	18	Pos	Pos	Pos	Pos	Pos
LT14/1490	Contact pig 12	18	Pos	Neg	Neg	Neg	Neg
LT14/1490	Contact pig 15	17	Pos	Pos	Pos	Pos	Pos

<sup>a</sup> dpi, days postinfection; dpe, days postexposure.

<sup>b</sup> Pos, positive; Neg, negative.

**IPT USED  
AS  
REFERENCE  
TEST**

Sample type	OIE-ELISA		Ingenasa-ELISA		IDvet-ELISA		Svanova-ELISA	
	No. of positive samples/total no.	Ss (%)	No. of positive samples/total no.	Ss (%)	No. of positive samples/total no.	Ss (%)	No. of positive samples/total no.	Ss (%)
Experimental	2/8	25	3/8	37.5	2/8	25	2/8	25
Field	2/10	20	6/10	60	4/10	40	4/10	40
Total	4/18	22.22	9/18	50	6/18	33.3	6/18	33.3

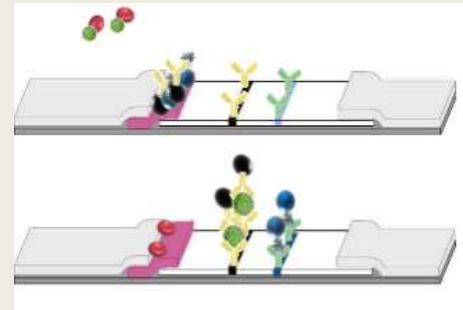


# New techniques for ASF diagnosis

## PENSIDE TEST

- Direct immunochromatography
- Monoclonal antigen vp72
- Blood
- Tested in DP and WB
- **Field conditions!!**

## INGEZIM ASF CROM ANTIGEN R.11.ASF.K42



the sensitivity of the assay respect to rt-PCR was 76% and the specificity 96%.



# New techniques for ASF diagnosis

## PENSIDE TEST

- Direct immunochromatography
- Antigen vp72
- Serum, plasma and full blood
- Tested in DP and WB
- **Field conditions!!**

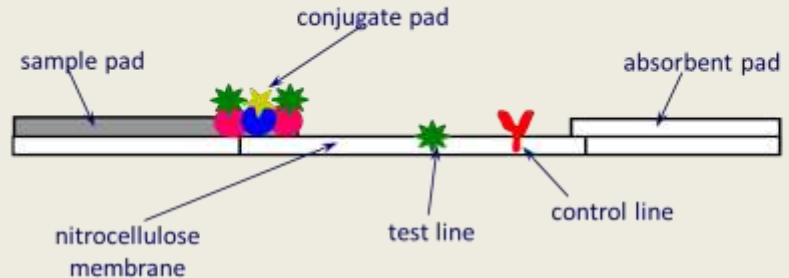
99% correspondence with the OIE ELISA. 82% sensitivity respect to IPMA (wild boars)





99,9% correspondence with INGEZIM® PPA COMPAC and OIE ELISA. 96% specificity respect IPMA (wild boars)

IPMA =  
IPT

## INGEZIM PPA CROM

### TECHNICAL BASIS & PRODUCT APPLICATION



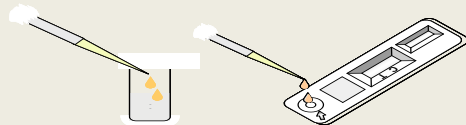
-  Test Line: VP72 protein of ASFV adsorbed on the nitrocellulose membrane
-  Control Line:  $\alpha$ -control protein MAb adsorbed on the nitrocellulose membrane.
-  Red latex microparticles covalently coated with VP72 protein.
-  Blue latex microparticles covalently coated with a control protein.



# New techniques for ASF diagnosis

## PENSIDE TEST

1. Add the diluted sample into the round window



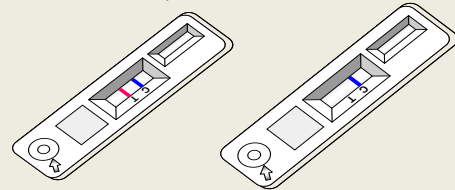
2. Read results at 10 minutes



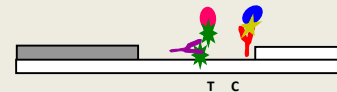
## INGEZIM PPA CROM

### TEST PROCEDURE

3. Interpretation of results



**POSITIVE  
SAMPLE**



**NEGATIVE  
SAMPLE**



capillary action



<http://www.sanidadanimal.info/cursos/asf/>



PROTOCOLS & VIDEOS



Thanks a lot  
Muchas

[jmvizcaino@ucm.es](mailto:jmvizcaino@ucm.es)

# **First Oral Immunization of wild boar Against ASF virus genotype II**

***TAFS forum. Scientific Conference on ASF  
Beijing, 2019***

**Prof. JM. Sánchez-Vizcaíno**

**Complutense University of Madrid  
Visavet Center**

***OIE-ASF Reference Laboratory***

***[www.sanidadanimal.info](http://www.sanidadanimal.info)***

***[jmvizcaino@ucm.es](mailto:jmvizcaino@ucm.es)***

# ASF:

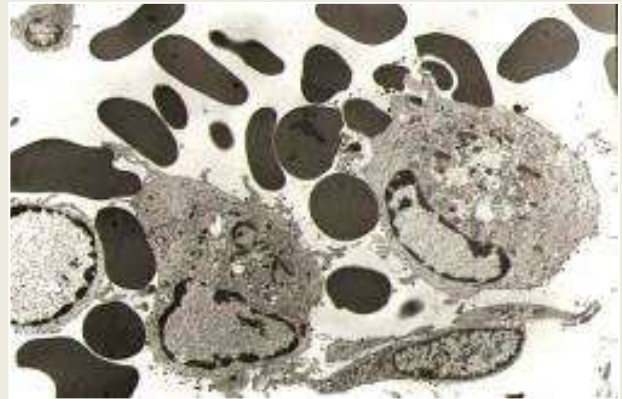
## *The most complex disease of swine*

Very complex  
molecular  
structure



More than 150 proteins;  
at least 50 proteins in the viral particle  
structure

Replication in macrophages, encodes  
multiple virulence factors



NO produces completely  
effective neutralizing  
antibodies. But is highly  
immunogenic



**Lack of effective  
vaccine**

**Lack of a complete protection**

# ***THE EXPERIMENT***

## **18 wild boar piglets:**

- 3-4 months old
- 10-15kg to 20-25 kg
- Origin: Cinegetic farm Lagunes S.L. CR, Spain.

## **2 strains :**

- Attenuated vaccine candidate strain (Lv17/WB/RIE1)
- Virulent challenge strain (Armenia/07)

**2 months**

***& 3 questions to answer***



1. Is attenuated strain able to generate immune response and no clinical signs nor mortality?
2. Is attenuated strain able to be transmitted by direct contact?
3. Does attenuated strain confer cross-protection against virulent challenge strain?



## ***The vaccine***

Attenuated ASF virus (ASFV)  
genotype II (non-HAD) from a wild  
boar detected in Latvia during  
2017 (**Lv17/WB/RIE1**)

## ***Oral vaccination***

$10^4$  TCDI<sub>50</sub> LV17/WB/RIE1

***+ In-contact vaccination***



Shedder-pig challenge-  
exposure infection model

## ***The challenge strain***

Virulent ASF virus (ASFV) genotype  
II (HAD) (**Armenia07**)



***IM challenge***

10 TCDI<sub>50</sub> Armenia07

***+ In-contact challenge***

# Sampling

2 times/week  
throughout the  
experiment

- EDTA blood
- Serum
- Fecal swabs
- Oral swabs
- Rectal temperature



- Viremia
  - ▶ Real Time-PCR
- Antibodies
  - ▶ ELISA Ingenasa
  - ▶ IPT
- Clinical scoring
- Post-mortem inspection

Clinical  
signs



# VACCINATION PERIOD

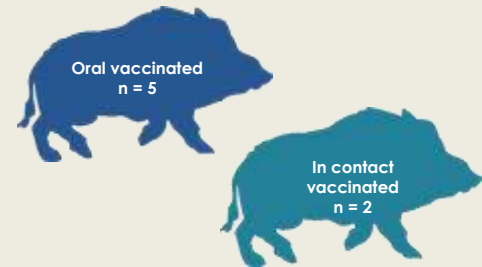
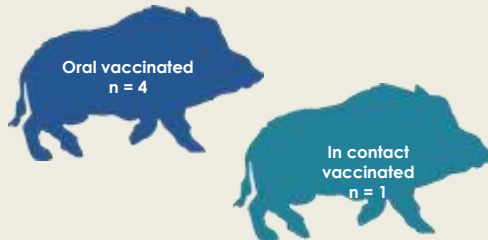
Day 0  
post-  
vaccination

Day 30  
post-  
vaccination

**BOX 1**

**BOX 2**

**BOX 3**



## RESULTS: VACCINATION PERIOD



**Slightly increase body temperature (40.1-40.8 °C) was the only clinical reaction detected (8/12)**



*Clinical scoring*

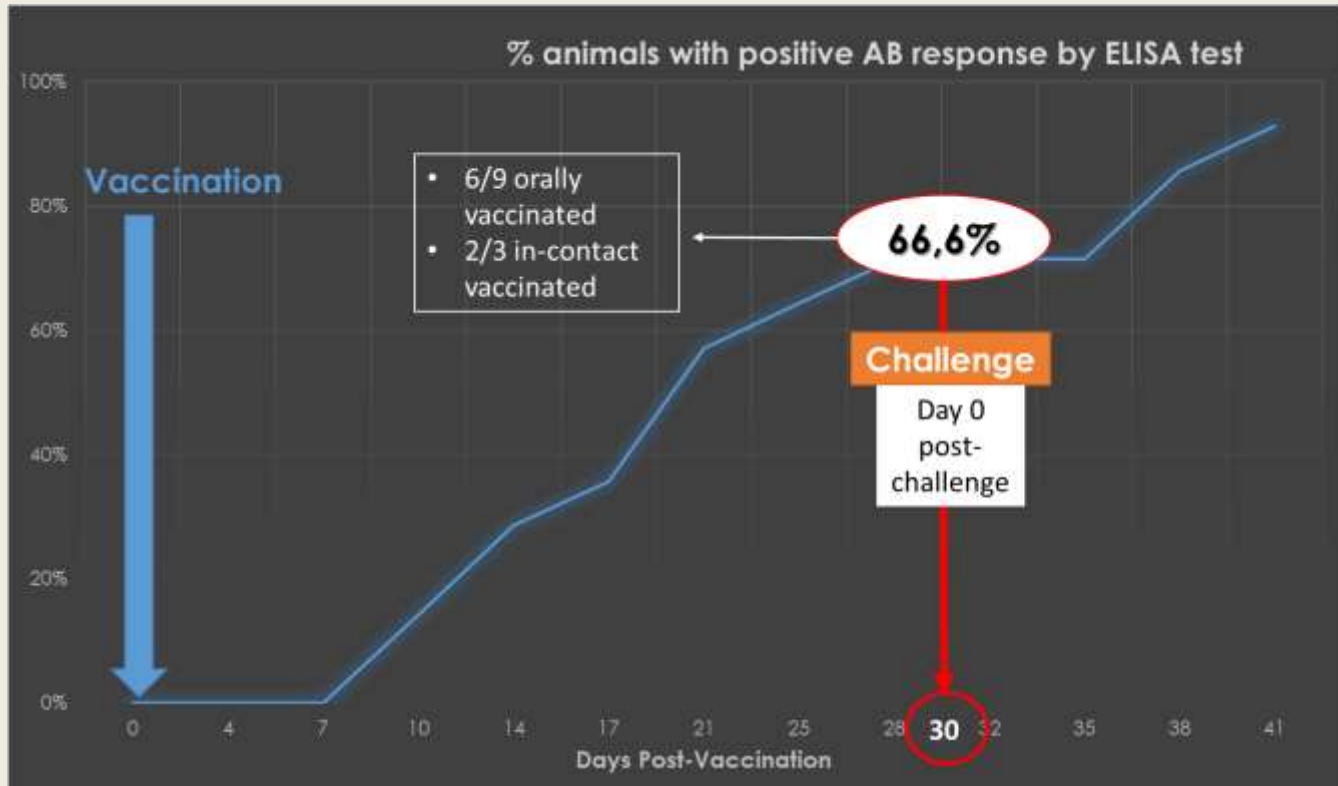


**Weak positive real-time PCR results ( $C_t = 33.02 \pm 4.07$ ) were sporadically detected (8/12)**



*Viremia*

# RESULTS: VACCINATION PERIOD



## Challenge by direct contact

IM Infection: 10 TCDI50 Armenia/07

## CHALLENGE PERIOD

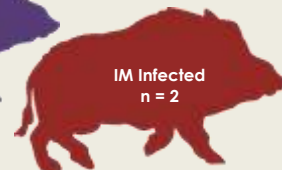
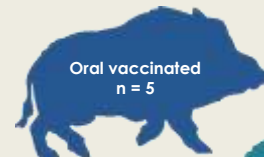
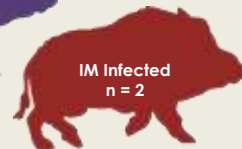
Day 30  
post-  
vaccination

Day 54  
post-  
vaccination

**BOX 1**

**BOX 2**

**BOX 3**

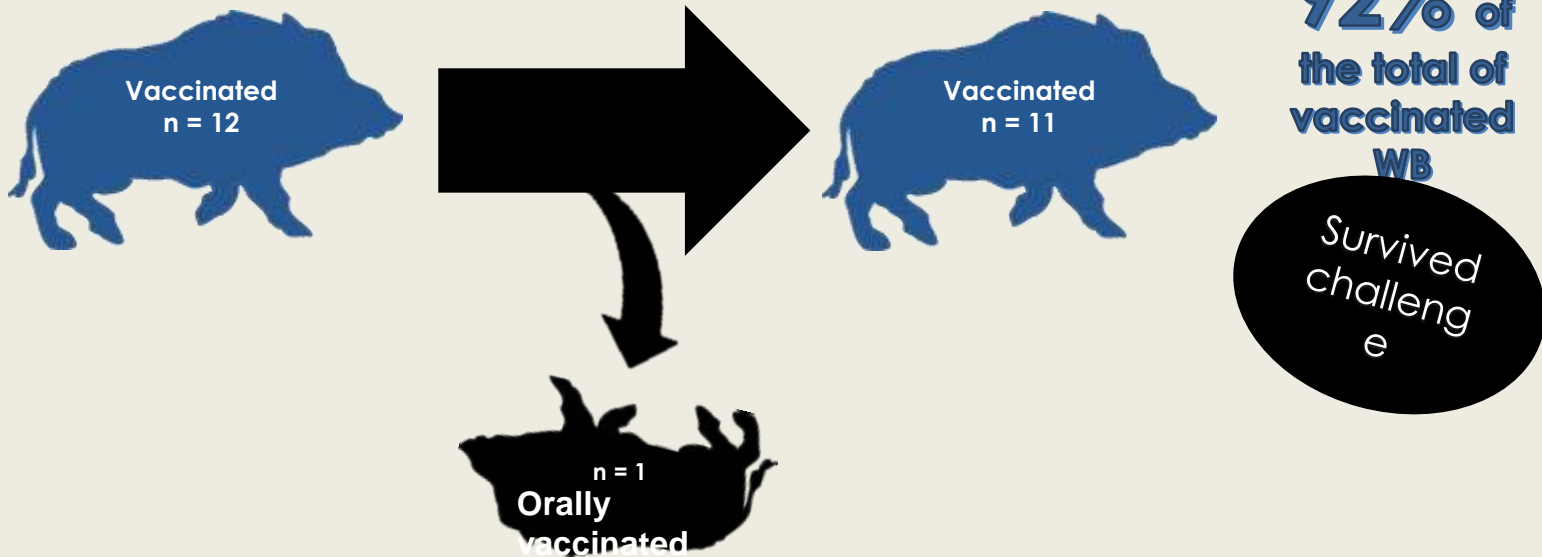


IM  
CHALLENGE  
D  
n = 1



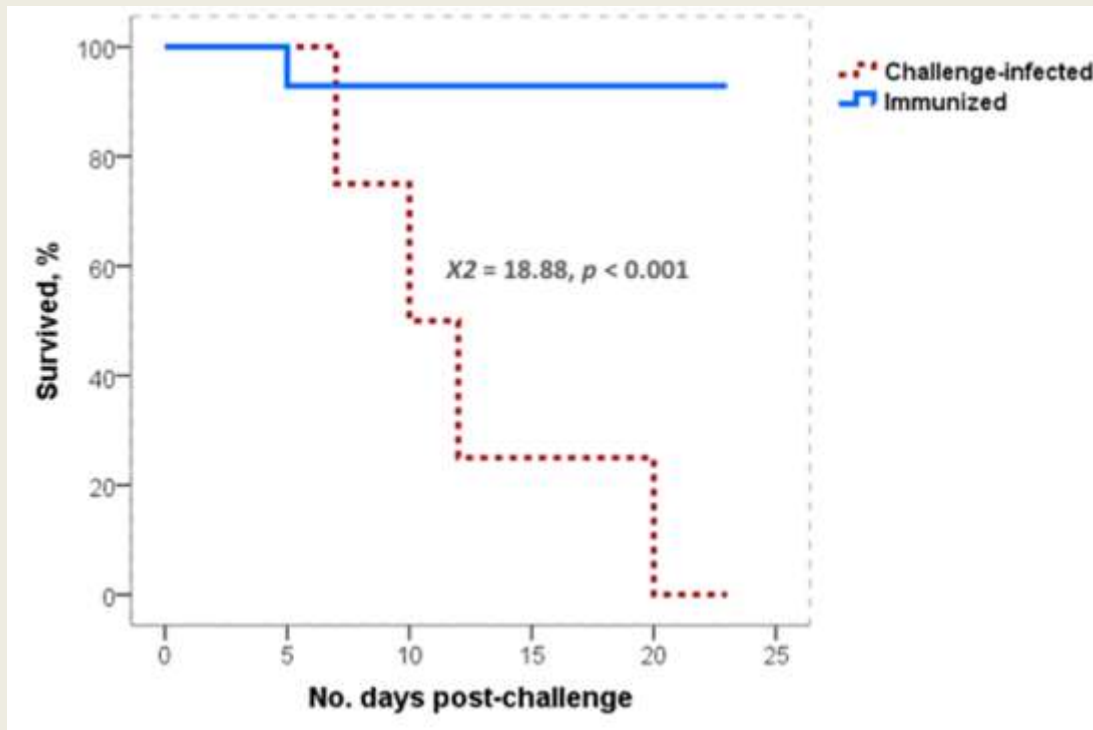
## RESULTS: POST-CHALLENGE PERIOD

### *VACCINE EFFECTIVENESS*



# RESULTS: POST-CHALLENGE PERIOD

## Immunized VS



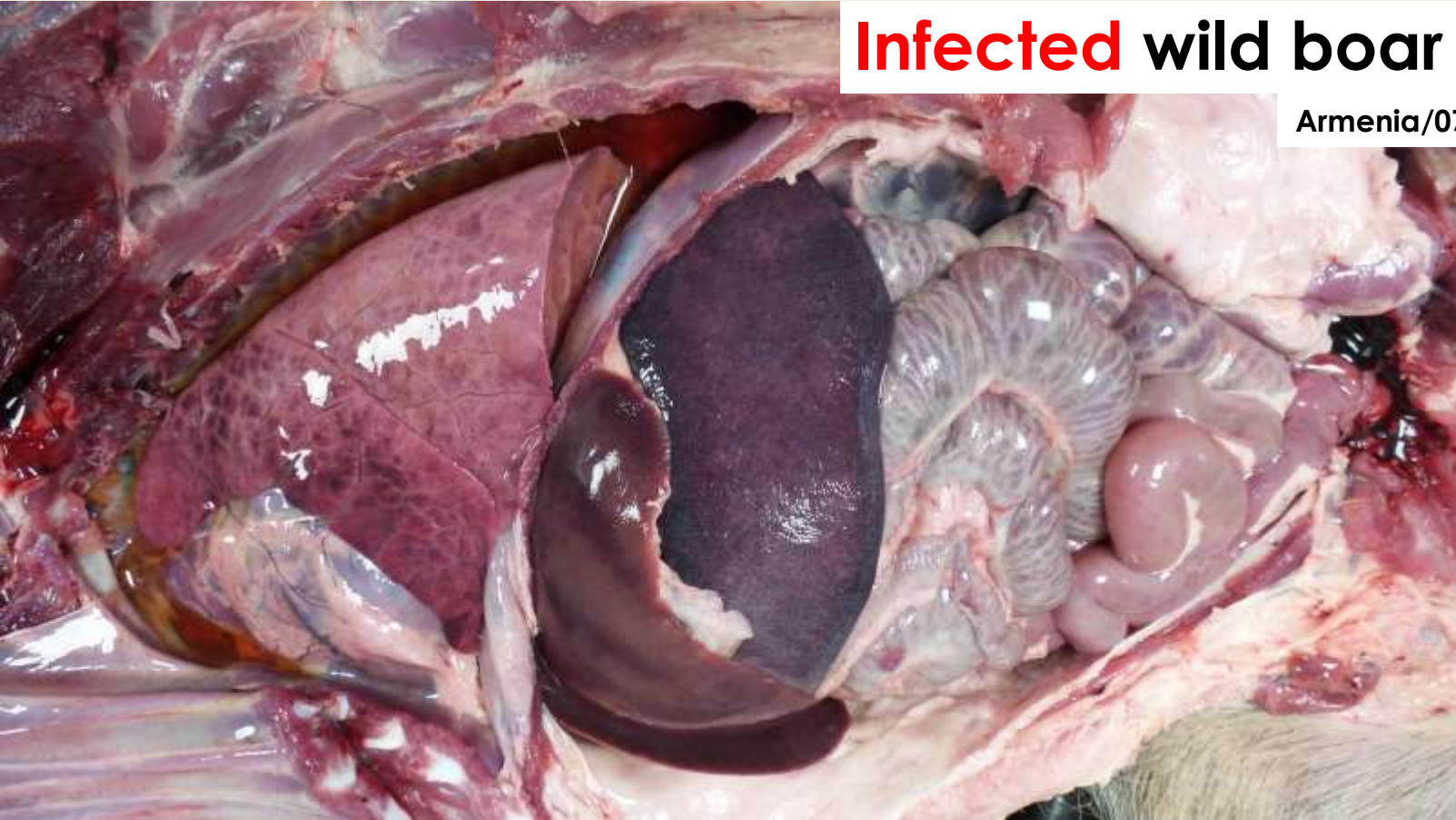
# POST-MORTEM STUDIES



# Immunized wild boar

Lv17/WB/RIE





**Infected** wild boar

Armenia/07

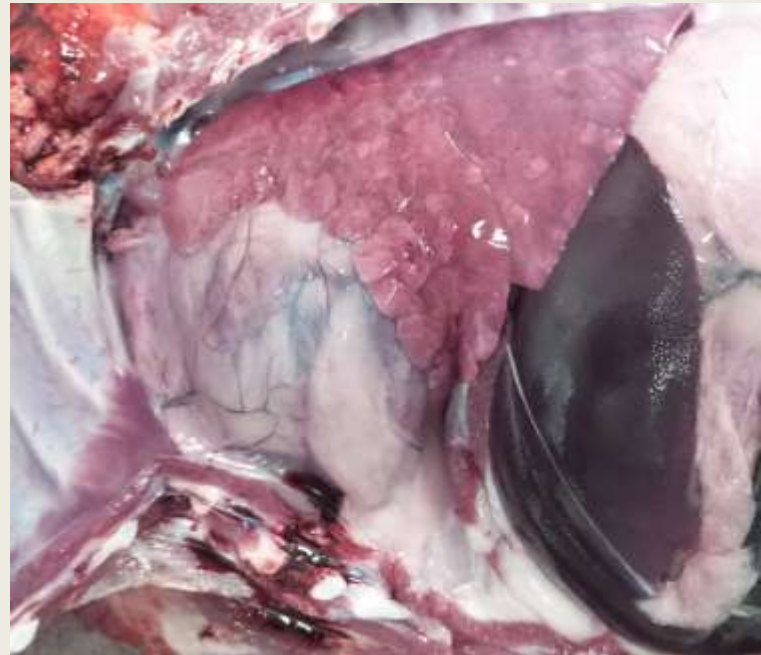
# Infected wild boar

Armenia/07



# Immunized wild boar

Lv17/WB/RIE1



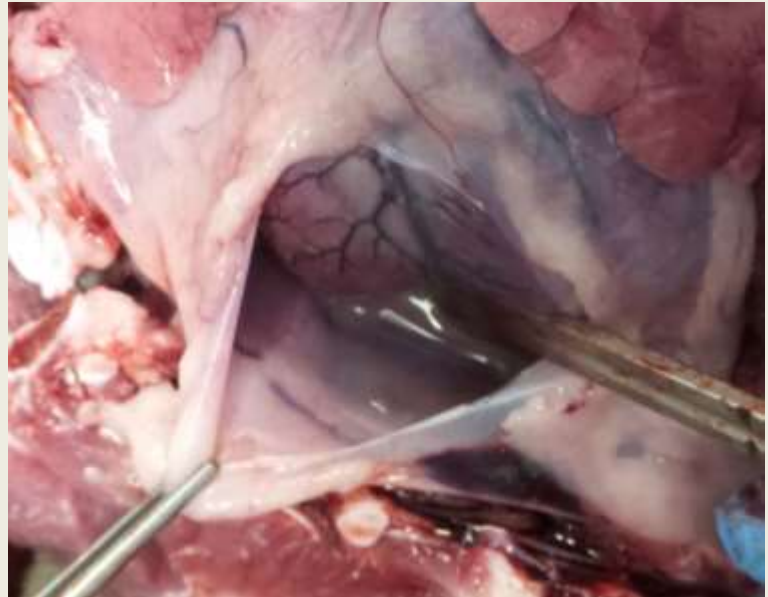
# Infected wild boar

Armenia/07



# Immunized wild boar

Lv17/WB/RIE1



# **Infected** wild boar

Armenia/07



# **Immunized** wild boar

Lv17/WB/RIE1



# **Infected** wild boar

Armenia/07

# **Immunized** wild boar

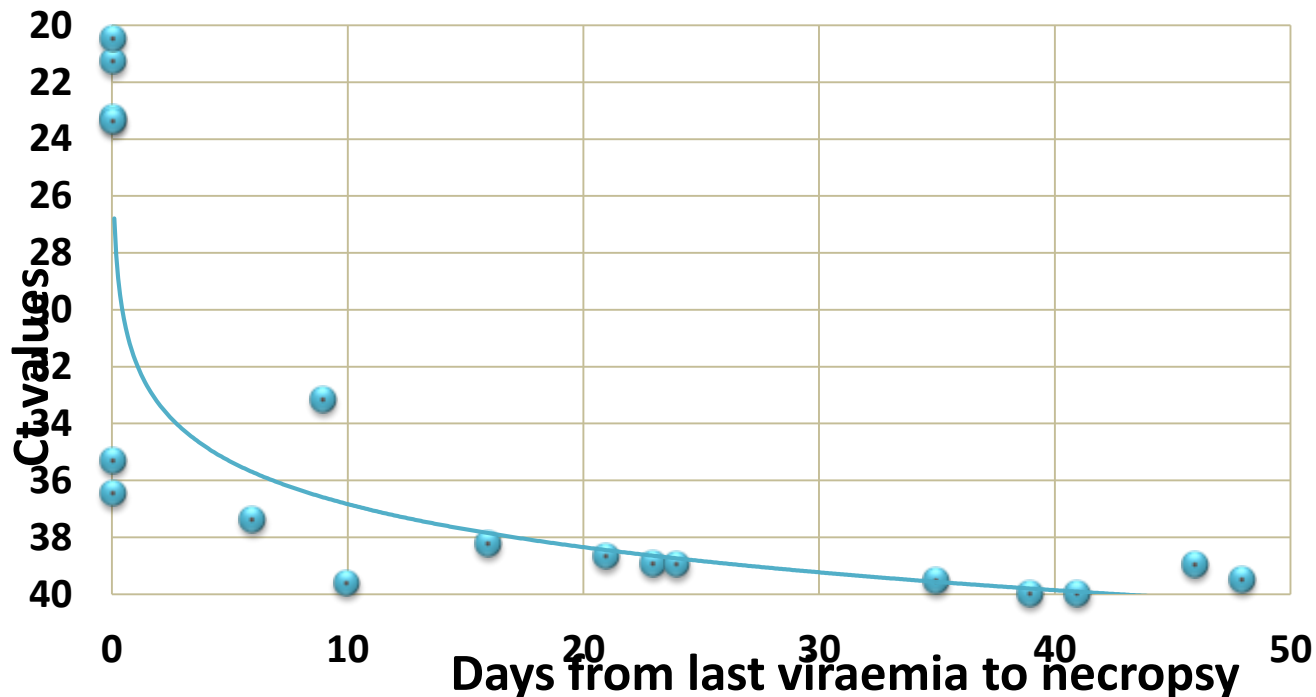
Lv17/WB/RIE1



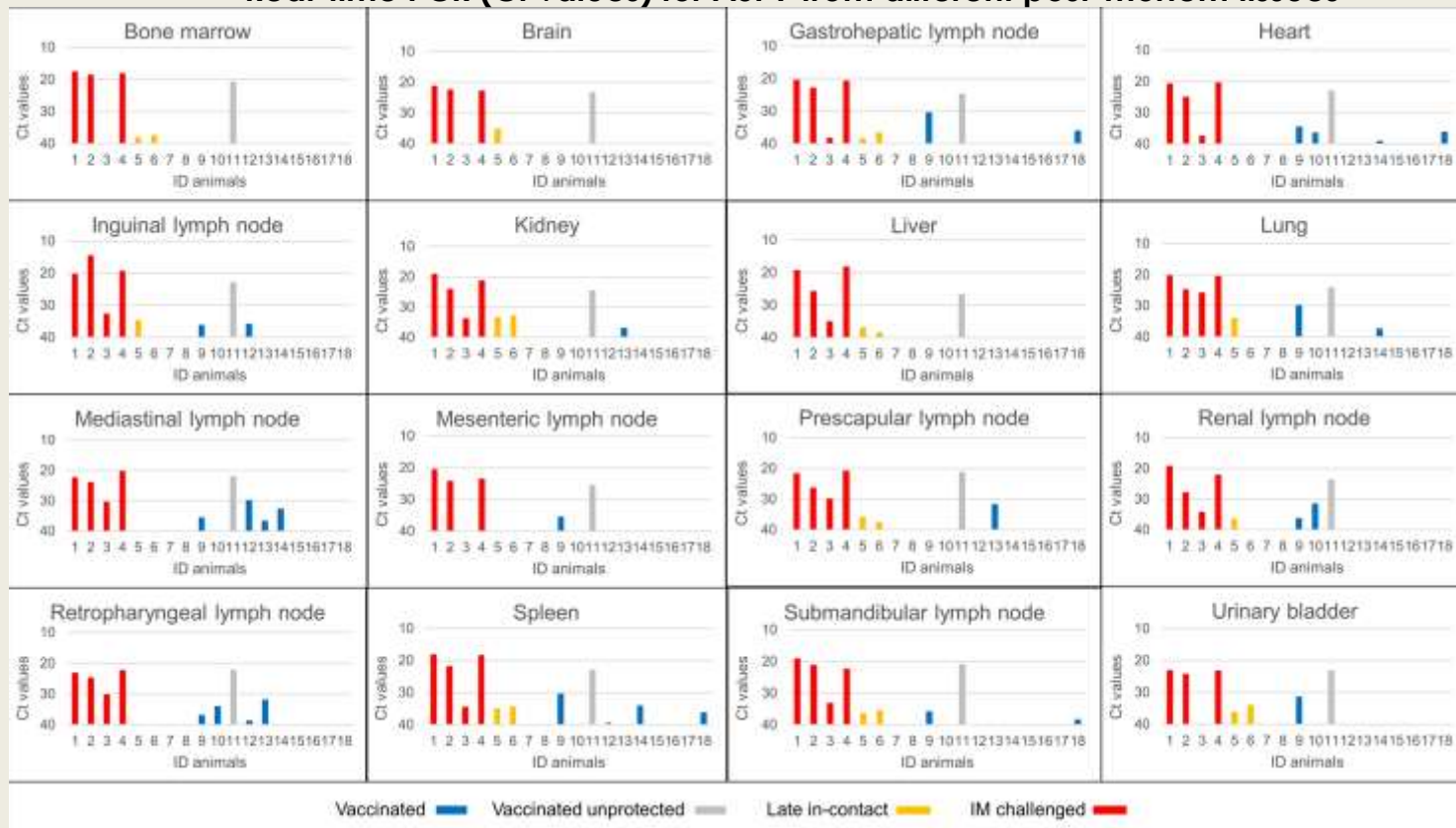
## POST MORTEN TISSUES EVALUATED by PCR

ANALYSED ORGANS (18)
Spleen
Kidney
Liver
Lung
Heart
Brain
Bone marrow
Bladder
Intestine
Tonsil
Submandibular lymph node
Inguinal lymph node
Retropharyngeal lymph node
Mesenteric lymph node
Gastrohepatic lymph node
Prescapular lymph node
Renal lymph node
Mediastinal lymph node

**Level of ASFV determined by real-time PCR from post-mortem tissues (n = 16) compared to the number of days from the last viremia detected to necropsy**

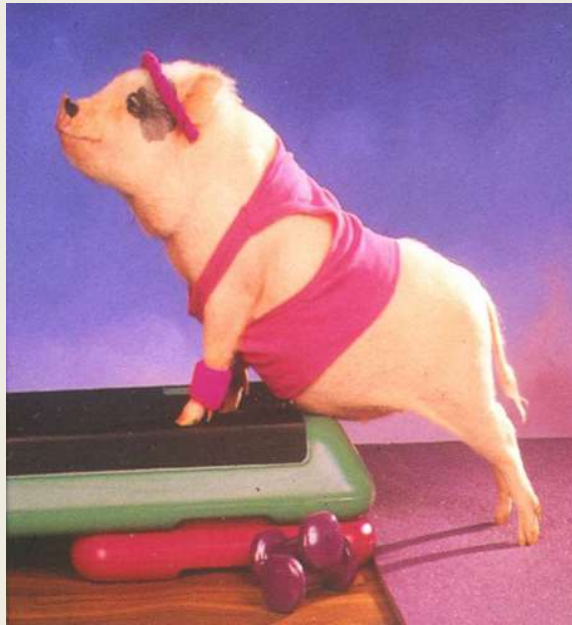


## Real-time PCR (Ct values) for ASFV from different post-mortem tissues



# VACCINE RESEARCH ON GOING

- Genetic stability in vitro and in vivo
- Overdoses immunization in WB
- Duration of Immunity (DP and WB)
- Immunization in domestic pig with a large number of animals
- DIVA adaptation



Thanks a lot  
Muchas  
gracias

[jmvizcaino@ucm.es](mailto:jmvizcaino@ucm.es)