REFERENCE LABORATORY

African Swine Fever

GREIFSWALD-INSEL RIEMS, APRIL 2007

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African Swine Fever

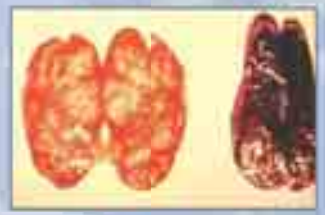




- Haemorrhagic disease of swine, belonging to the former OIE List A diseases
- It causes great economic losses in affected countries.

High mortality rates associated with acute and peracute forms
Estrict sanitary measures for control and eradication

Incubation period range: 4-19 days.



Acute forms, 90-100% mortality

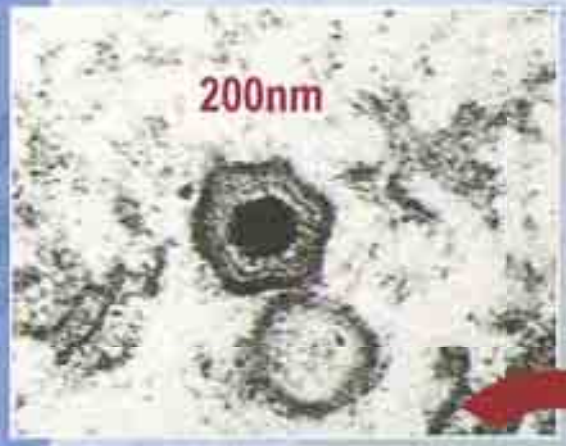
African Swine Fever

ASF only affects PORCINE SPECIES

both wild host and domestic

- **Domestic pigs and European wild boars are very susceptible to infection**
  Animals that survive to infection could become into asynthomatic carriers.
- **Wildlife hosts in Africa are persistently infected for long periods with no disease signs.**
- **soft ticks of the *Ornithodoros* species, O.erraticus (Europe) and O.moubata (Africa) acts as reservoirs and transmission vectors of ASFV**

African Swine Fever



- Icosahedral complex DNA virus , 170-190 kbp
- Enveloped virus
- Only member of the Asfarviridae family (derives from "ASF and related virus").
- Similar replicative system than Poxviruses.
- No serological serotypes can be discerned.

Virus isolates vary in pathogenicity - high and low virulence forms -

Main Target Cells.:
Monocyte/Macrophages

Attachment of erytrocytes to the external membrane of ASF-infected macrophages

Virus replication also observed:

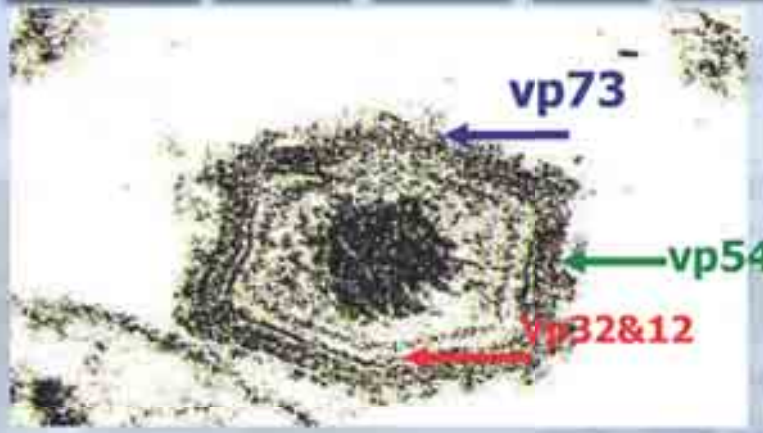
- Endothelial cells
- Hepatocytes
- Renal tubular epithelial cells
- Neutrophils cells

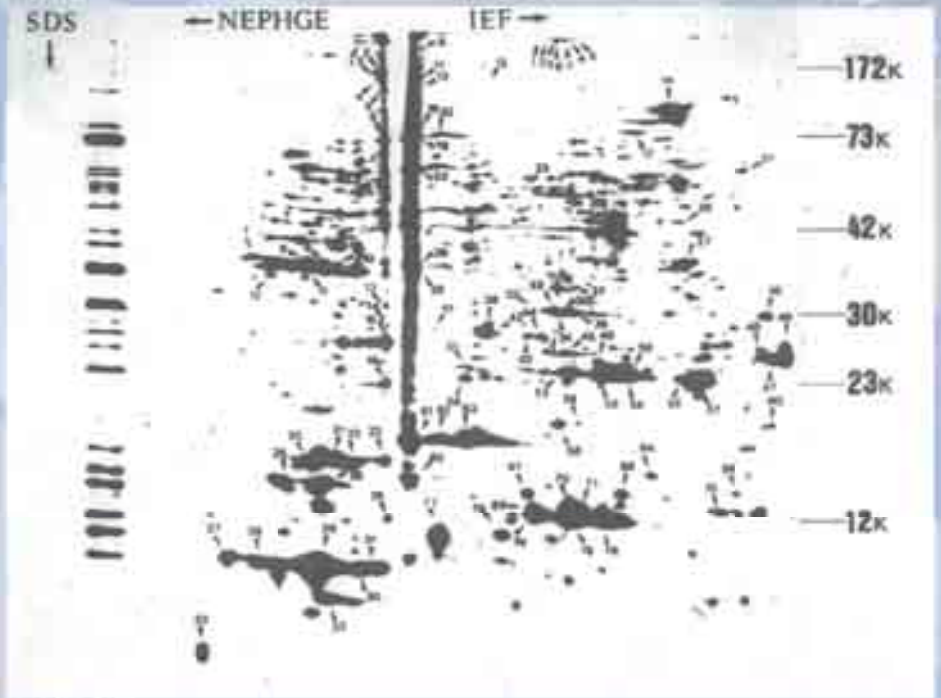
Few ASFV isolates do not produce haemadsortion effect.

ASF VIRAL PROTEINS

- 28-34 structural proteins
- 95-111 infectious proteins

Immunogens: >50





PROTEINS OF MAJOR IMPORTANCE FOR DIAGNOSIS

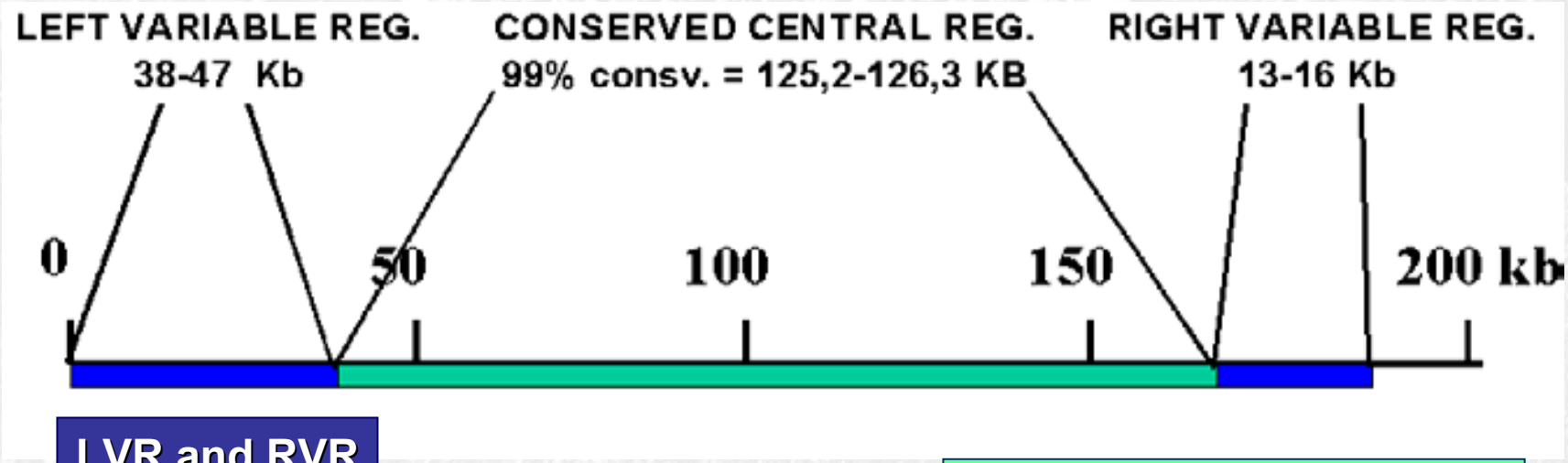
- P72
- pp62
- P32 (30)
- P54
- p35-23.5
- P12

induce antibodies in early stages of infections

AFRICAN SWINE FEVER VIRUS GENOME

- 170-190 Kbp. Encodes about 150 ORFs. Complete analysis of the ASF genome sequence (1 europe, rest from Africa)

Genome variability mainly due to deletions and insertions



LVR and RVR

- Encodes 5 Multigene families

MGF100:	2-3 copies per genome
MGF110:	5-17 copies per genome
MGF300:	3-4 copies per genome
MGF360:	11-19 copies per genome
MGF505/530:	8-10 copies per genome

-Large length variations between 3-20 kb,
Due to deletion and insertions of complete genes

↓

Restriction Fragment Length polymorphyms
(by RE studies Eco RI, HindIII…)

Conserved Central Region

Tandem Repeat Sequences (TRS)

↓

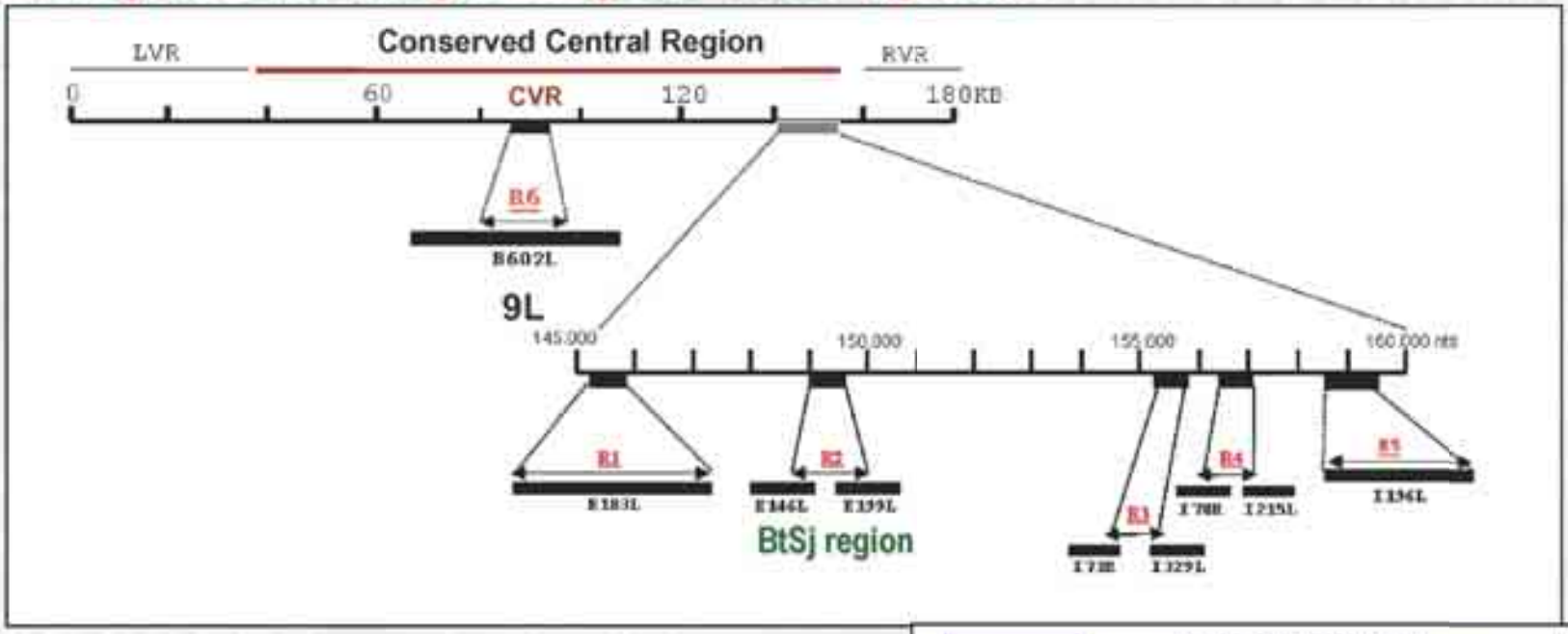
-Small length variations < 1kb (
(Deletion and insertions of TRS)

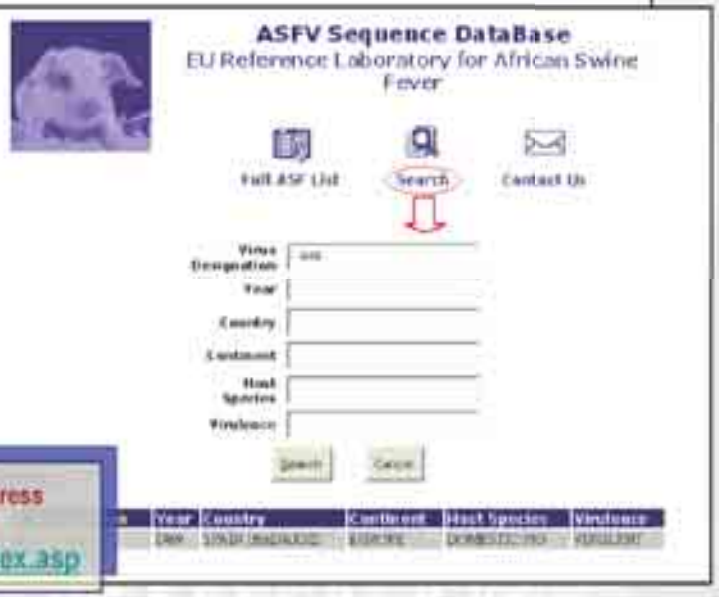
↓

PCR fragment size and Sequencing

GENETIC CHARACTERIZATION OF SPECIFIC SEQUENCES
ONLY METHOD TO ESTABLISH RELATIONSHIPS AMONG DIFFERENT ASFV ISOLATES

MOLECULAR EPIDEMIOLOGY : Sequence analysis of tandem repeats in different genes and intergenic regions of ASFV genome





The web database is located in the address

http://webainia.inia.es/cisa/asfv/index.asp

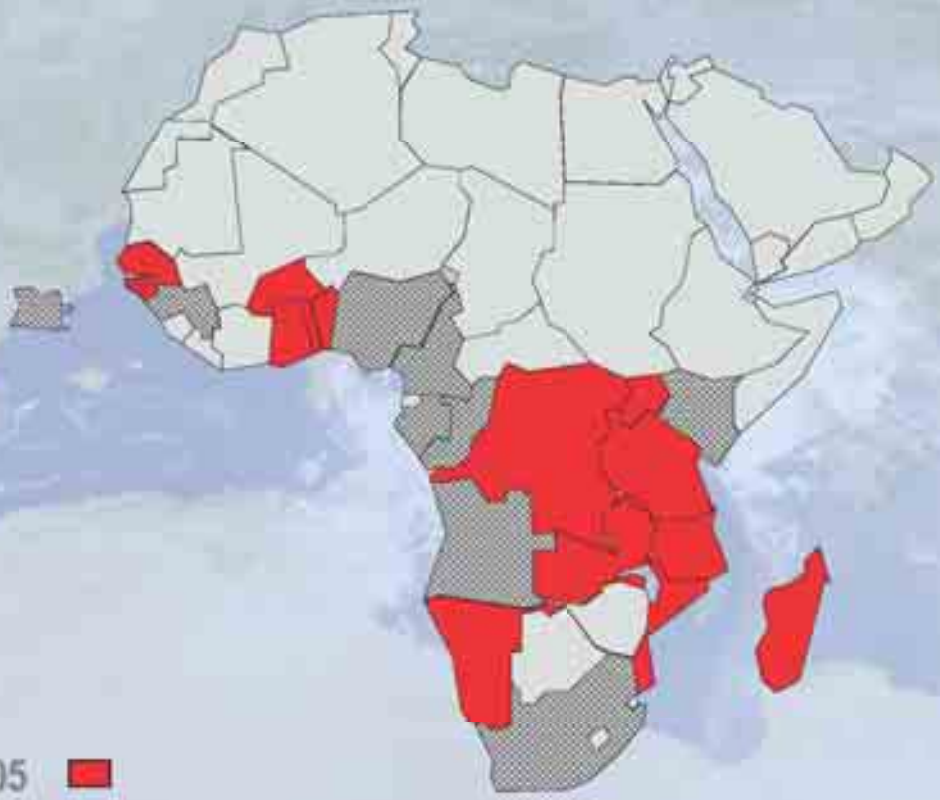
African Swine Fever

Eradicated in the Iberian Peninsula, 1995

African Swine Fever

1997-2002

OVERVIEW OF ASF IN AFRICAN COUNTRIES (2000-2005)



OUTBREAKS 2004-2005

OUTBREAKS 2000-2003

SOURCE: World Organization for Animal Health (OIE)

African Swine Fever

Current ASF distribution in Africa

Endemic

- Senegal
- Gambia
- Guinée Bissau
- Burkina Faso
- Togo
- Benin
- Nigeria
- Cameroon
- Congo Brazaville
- Angola,
- Cabo Verde
- RDC
- Uganda
- Rwanda
- Burundi
- Kenya
- Tanzania
- Mozambique
- Namibia
- Madagascar

Under control

- Ivoiry Coast (eradicated)
- Ghana
- Sao Tome et Principe

SOURCE: World Organization for Animal Health (OIE)

Eastern and Central-Southern Africa

Phacochoerus africanus,
Warthog

Known extent of occurrence

VIRUS MAINTAINED IN AN ANCIENT SYLVATIC CYCLE:

Wild suids become infected while very young through the bites of infected o.ticks

in burrow

Potamochoerus porcus, Bush Pig

-Low levels of virus in tissues
-Transient viremia- low or undetectable -
no clinical disease

NO EVIDENCE THAT THEY CAN INFECT DOMESTIC PIGS DIRECTLY

Some evidence: Diseased bushpigs could transmit ASFV

Western Africa

Phacochoerus aethiopicus,
Red River Hog

Known extent of occurrence

No tick vector is known in the western Africa.

It is not clear whether any tick and wild life host play a role.
Wilna Vooslo, 2007

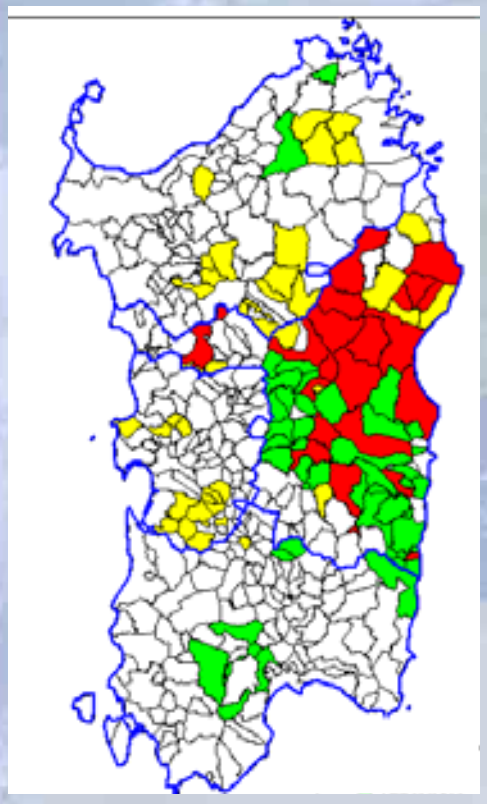
Hylochoerus meinertzhageni,
Giant Forest Hog

Main source of transmmision possibly.:

By direct contact between sick and healthy animals

By eating infected swill or garbages containing uncooked infected meat pork

COMMUNES OF SARDINIA WHERE ASF OUTBREAKS OCCURRED SINCE 1993



1993-2003 (37)

2004-2005 (38)

1993-2005 (20)

Pig Breeding system (herds -17.774-) in Sardinia

Backyard (16,031) : 90.34%

Intensive (320): 1,80%

Confined/Fr (899): 5.06%

Free ranging (494).: 2.78%

No tick vectors in Sardinia

Pig population.: 248.356

Epidemic weaves: 94-96; 2004-2005.

Source of info.: NRL for ASF meeting , 2006, D. Rutili

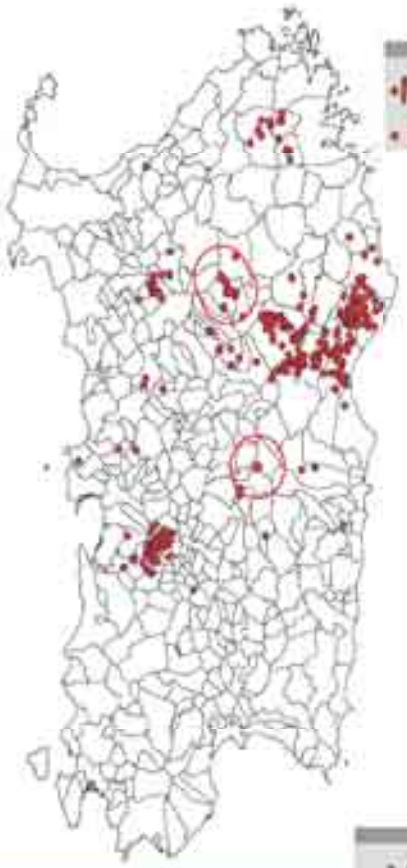
African Swine Fever





Epidemiological units of wild boar in Sardinia

ASF outbreaks occurred in domestic pig (red dots) and in wild boar (red rings) during the period 2004-2005



- Most of outbreaks in backyard pig farms
- In most of the cases no enough inf. to explain the origin.

Even if Wild boar play an important role as reservoirs of ASFV, it seems that they have not been a critica lrisk factor in the spread of ASF in Sardinia

Serological control of wild boars during hunting season 2005/ 06:
Sample tested : 4.194.
Prev. : 2,17%. Virus +ve: o%

Main factors that are contribuiting :

- uncontrolled pig movement and free-ranging pigs.

- Vet. Services: difficulties to deal with the ASF emergengy involving a large number of medium and little size herds spread all over the territory

Main Routes of Transmission and Dissemination





By direct contact between sick and healthy animals (exudates, wounds,...)

By eating infected swill or garbages

ONCE ESTABLISHED

CARRIERS PIGS play an important role in the dissemination of the disease

To be a major consideration in designing strategies for ASF eradication

Contaminated transport

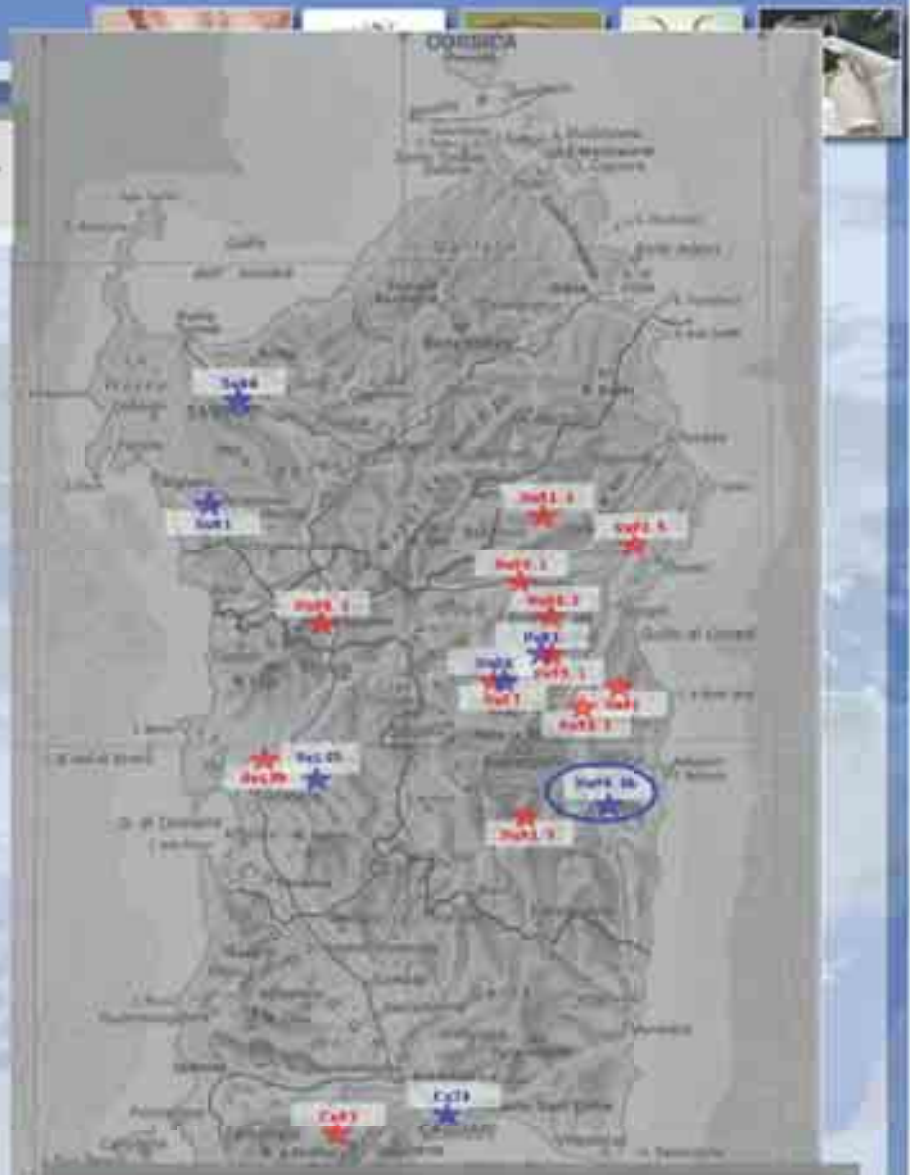
Contaminated clothes and shoes, material in farms...

slurry, insects, rats...

African Swine Fever

Major ASFV groupings/genotypes identified in the B602L region (R6-9L-) analyzed.

GENOTYPE	ORIGIN	ISOLATES	TYPE OF TRS
I	Europe	BA71V: Por63	KADNAAAAAAAACDNADNADPCADNTDDCAPAY
II	Europe	Ca78;Nu81;Es81;Ors84; Or185;Ba88;Nu90.88; Al161;Av71;E874;E75; Nu82;I85;Ba88;De88; Nu90;Nu94;466;Fr64; Ba192	KADNAAAACDNADNADNADNTDDNAPAY
III	Europe (Sardinia)	Nu90;Nu90.2;Or190; Nu91.3;Nu91.5;Nu95.1; Nu96;Ca97;Nu97;Nu98.9	KADNAAADDNAPAY
IV	Europe (Spain)	Ms1 Co62	KADNAAAAAAADADNADNADNTDDPAY
V	Europe (Spain)	Co61	KAAADNAAAAAAAACDNADNADNADNTDDNAPAY
VI	Europe (Spain)	Co68	KADNAAAAACDNADNADNADTDDNAPAY
VII	America	Bc70	KADNAAAACDNADTDDNAPAY
VIII	America	BA70	KADNAAAACDNADNADDDDNAPAY
IX	Africa	Mz66	KADNAAAaDDDNNADNADnDnAnY
X	Africa	Kat57	KAAAAAAAADNADTDDNAAAAAAAY
XI	Africa	Ang72	KAADNADTDDNAAAAY
XII	Africa	Cam82	KADNAAAACNADTDDNAAAAANAY
XIII	Africa	CV97; CV98	KAADNADNADTDDNAAAAY
XIV	Africa	Nig01	KAAACDNAAAAAACDNAAAAAACDNDAAAACDNAY
XV	Africa	Ug03N;Ug03F	KAAAADNADDNADDDDDDNADDNNAY
XVI	Europe	Ba178	KADNAAAAADNADNADNADNADTDDNAPAY
XVII	Africa	MW Lil20/1	KAVDVDGDNAVNDGVVVVDGNAVNDGVVNDGVDGVY



B602L genome region: Isolates from Sardinia were placed together in two groups.: 1978-1990; 1990-to date.

Clinical diagnosis

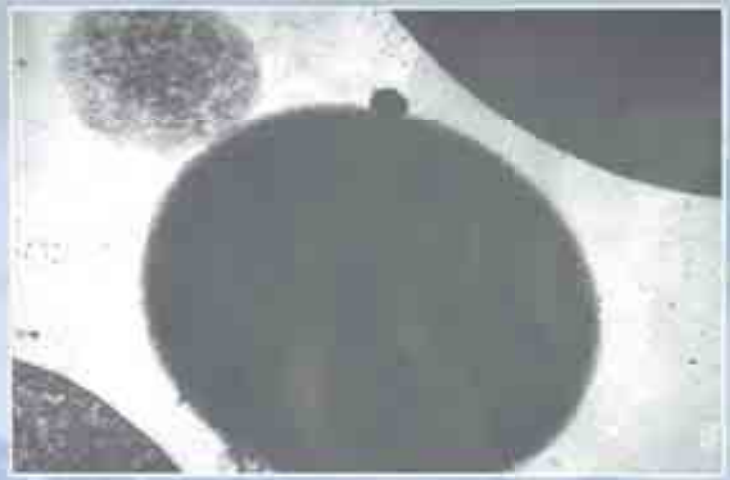
- ✓ African Swine Fever
- ✓ Classical Swine Fever
- ✓ Erisipelas
- ✓ Salmonellosis
- ✓ Other Septicaemic conditions
- ✓ PDNS

ASF 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 7-12dpi.

Abs good infectious marker¡¡

- Antigen – Antibody Immunocomplex Formation

Low sensitivity in the antigen detection techniques

SAMPLE COLLECTION





WHOLE BLOOD
SERA
FTA CARDS
TICKS
TISSUES
- Spleen
- Liver
- Lung
- Kidney
- Mediastinic L.N.
- Renal L.N.
- Retropharingeal L.N.

ASF LABORATORY DIAGNOSIS

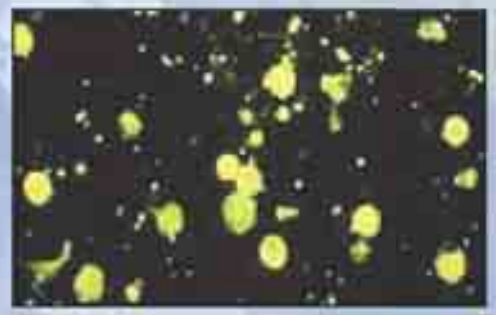




- ***Identification of the Agent***
  - *Isolation in cells cultures*: **Haemoadsorption'autorosette' (HA) test** with peripheral blood leukocytes from infected pigs



  - ***Direct immunofluorescent test*** (DIF)
    - Low sensitivity in subacute and chronic forms



  - Antigen ELISA

Significant lack of sensitivity due to Ag-Ab complex formation. Not recomended for analysis of serum and tissue –homogenated samples after first week pi. due to false negative results.

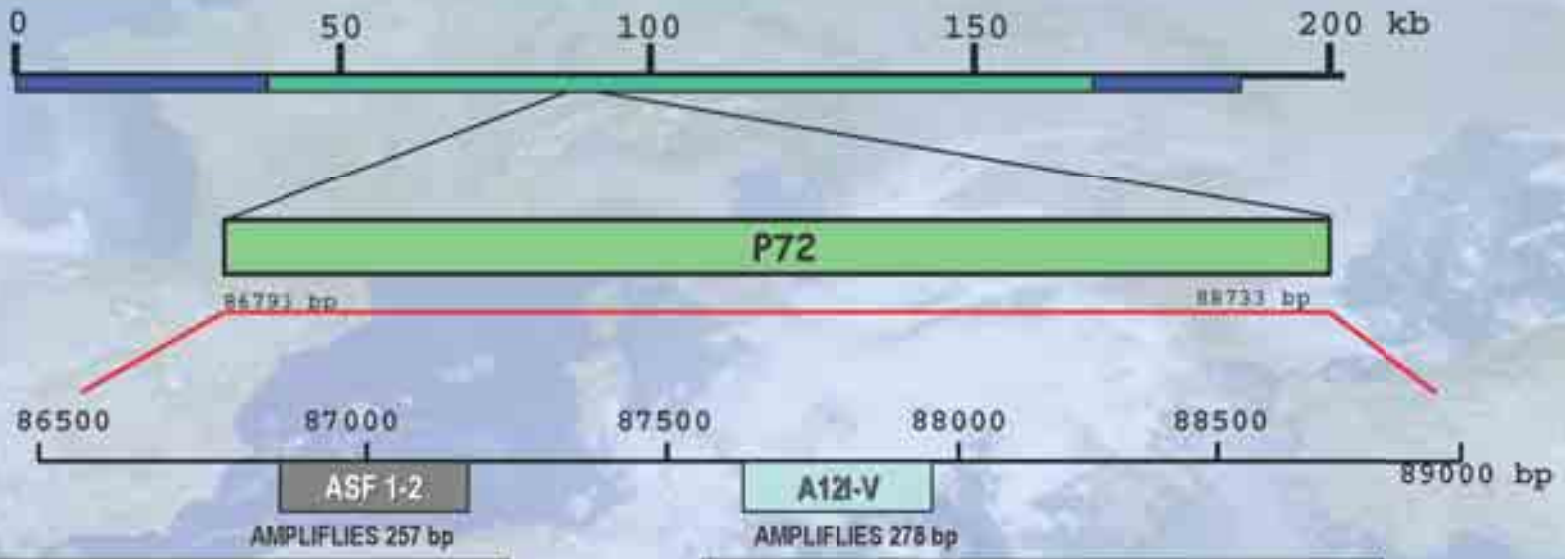
ASF LABORATORY DIAGNOSIS





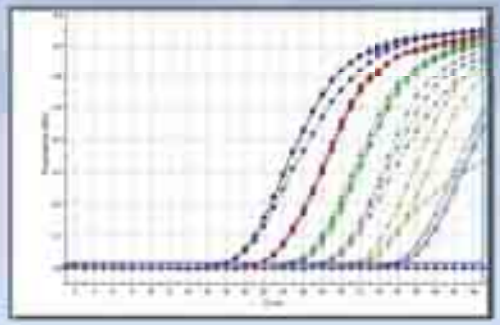
Virus detection by PCR

PCR DETECTION USING DIAGNOSTIC PRIMERS



. Aguero M, Fernandez J, Romero L, Sanchez Mascaraque C, Arias M, Sanchez-Vizcaino JM. J Clin Microbiol. 2003 Sep;41(9):4431-4.

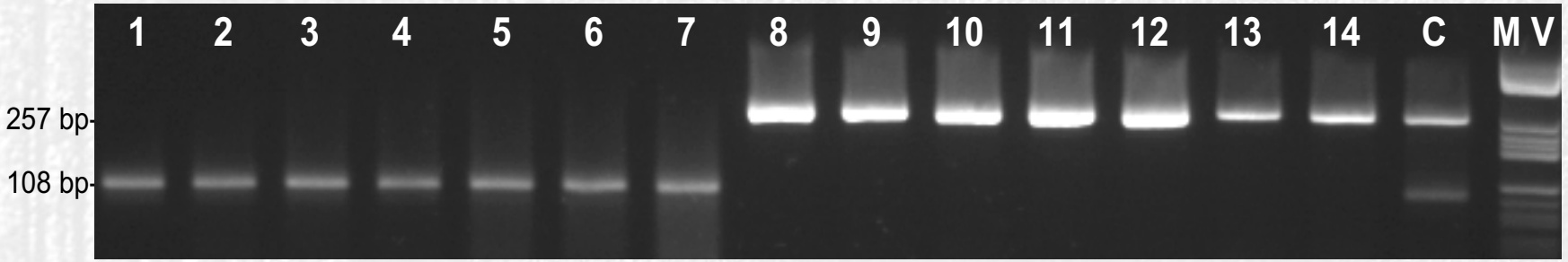
In OIE Manual of standards for diagnostic test and vaccines, Wilkinson, et al. 2004



Real time King et al, 2003,

MULTIPLEX CSFV/ASFV RT-PCR ASSAYS:

CSFV and ASFV Diferential Detection in clinical samples



PPC-3/4 + PPA-1/2

3% agarose gel

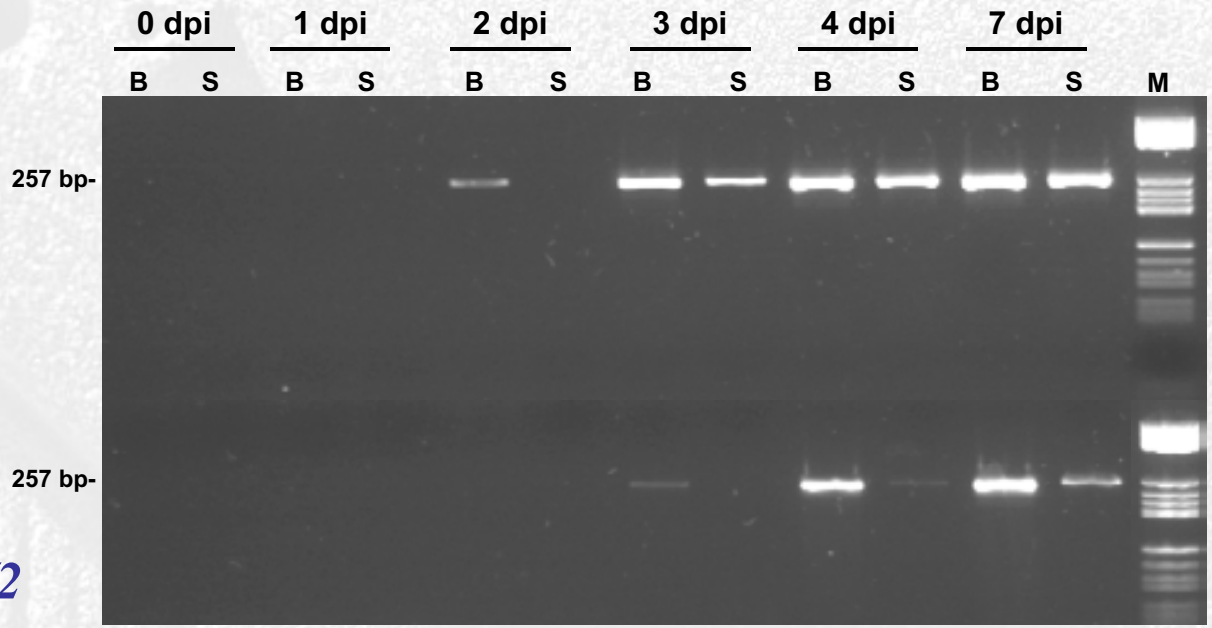
- **1-7: Samples from Spain2001 CSFV infected pigs. 1 and 2: Serum, 3: Blood, 4: Kidney, 5: Lymph node, 6: Spleen, 7: Tonsil.**
- **8-14: Samples from a Spain70 ASFV experimentally infected pig. 8: Blood, 9: Spleen, 10: Kidney, 11: Lymph node, 12: Serum, 13: Liver, 14: Tonsil.**
- **C: Multiplex PCR positive control.**

***ASF* Sensitivity : 0.12 HADU50/PCR for *I*ndividual PCR and 1.2 HADU50/PCR for *M*ultiplex PCR.**

***Multiplex PCR*, CSF sensitivity: 0.32 TCID50/PCR**

Individual ASFV PCR/Multiplex CSFV/ASFV RT-PCR Viraemia Study for ASFV

Pig inoculated with 10^4 $UHAD_{50}$ of Spain 75 ASFV strain



PPA-1/2

PPC-3/4 + PPA-1/2

ASFV **detected in blood at 2nd/3rd day post-inoculation, in serum at 3rd/4th day post-inoculation.**

ASF LABORATORY DIAGNOSIS





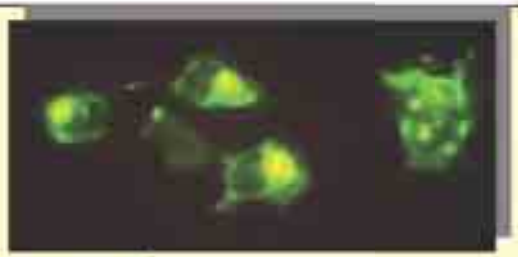
Antibody Detection

- **ELISA tests**

  - Indirect ELISA (OIE)
  - In House ELISAs
  - Commercial ELISA, Ingezim K3


Sensitivity with ASF-positive field samples: 100%; carriers: 77,7%; specificity : 100% . pre-treatment (hot-treatment at 56°C, 30 minutes), does not affect the final results .

- ***Indirect immunofluorescent test*** (IIF)



- ***Inmunoblotting (confirmation test)***



What is used in Europe?

Ring Trial 2005/06

19 out of 25 European countries (17 from EU) performed the Ring test.

Antibody Detection Techniques

in 2004: 80%

ELISA test: 90%. 5 labs performed "in house" or OIE test.
Rest of them used Commercial ELISA

in 2004/05: 66%

Confirmation techniques:
IB: 90% NRLs. 40% failed in the limits
IIF 1; IIP: 1

Viral Detection Techniques

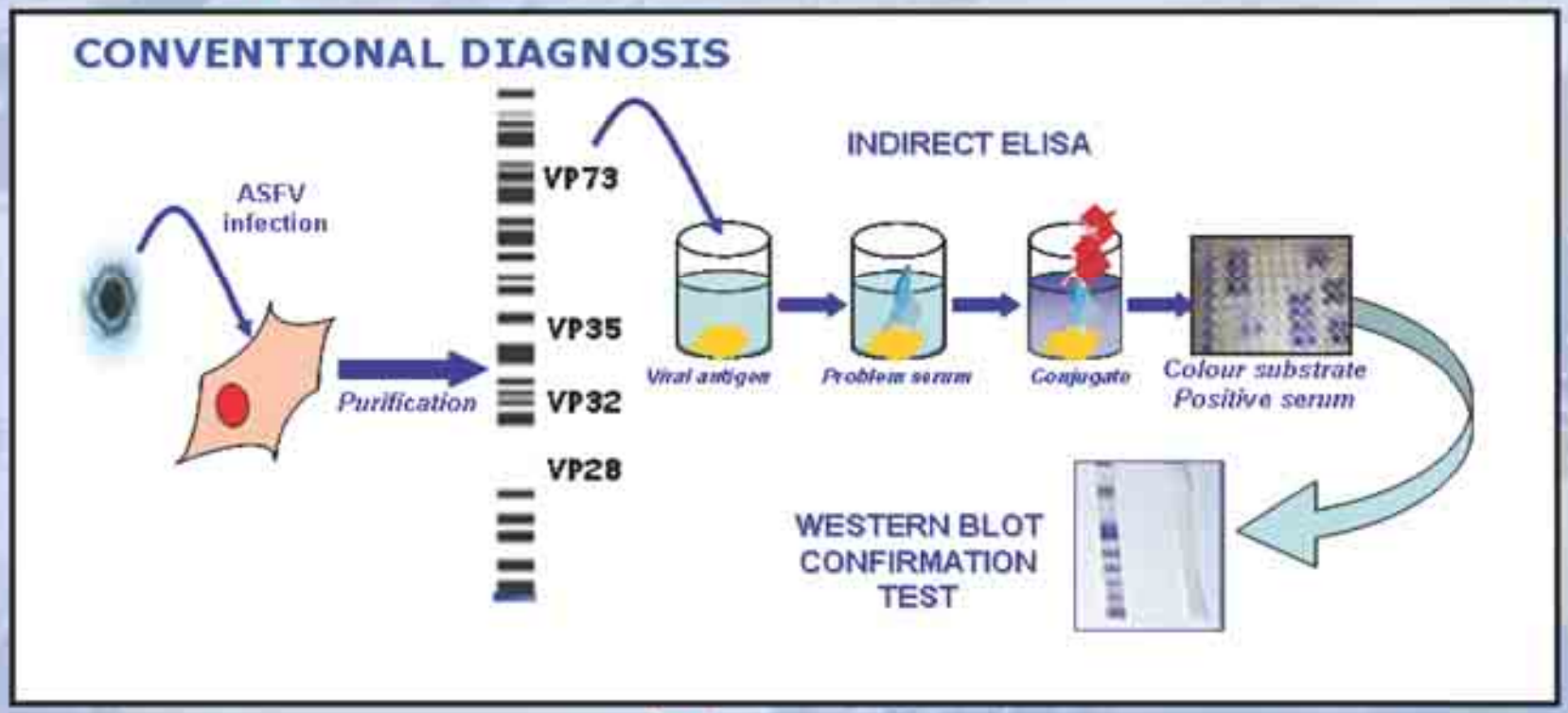
in 2004/05: 33%

- Viral isolation was performed by 48% of NRLs.

in 2004/05: 60% -4 new NRLs-

- PCR test was performed by 76% of NRLs.
- Antigen commercial ELISA test performed by 23% of NRLs (3 from the 5 didn't run PCR).

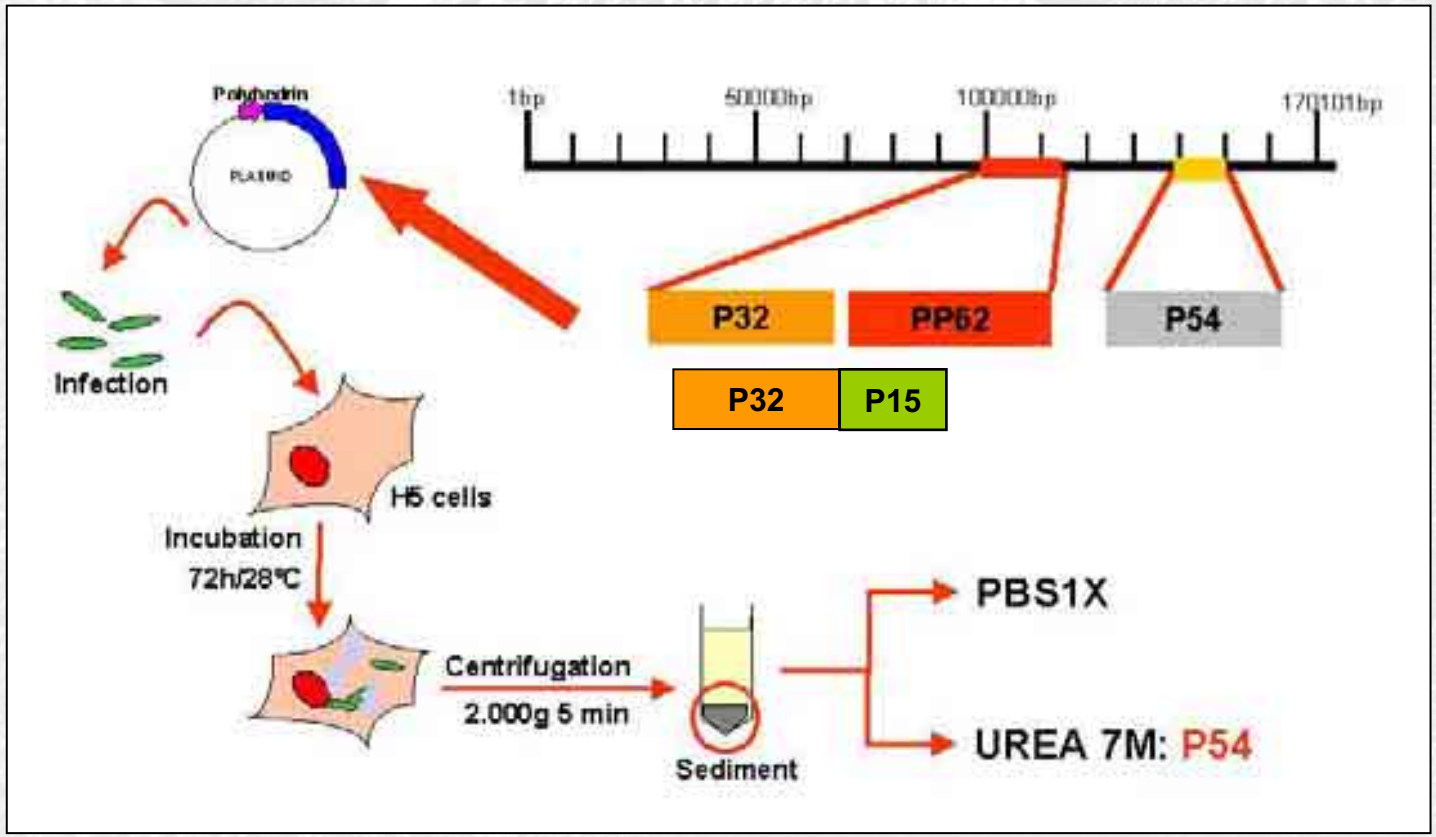
Antibody Detection

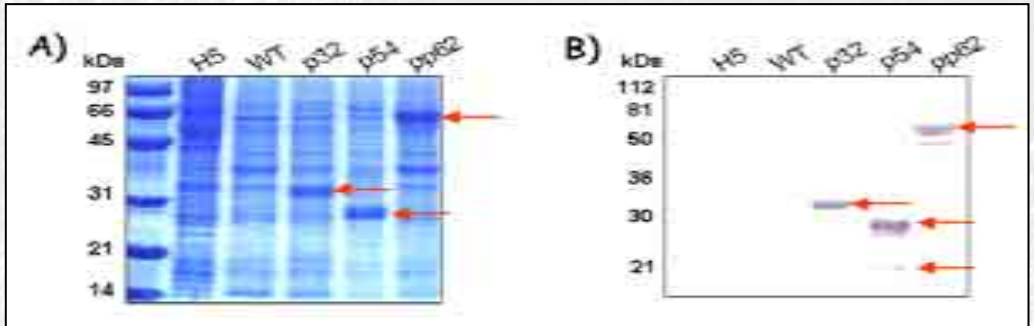


i) Use of **infectious virus** in the antigen production

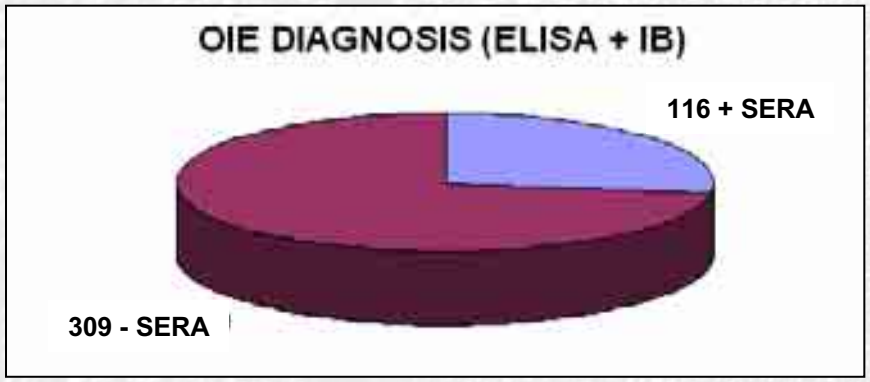
ii) **Loss of sensitivity** for the analysis of samples badly preserved samples increasing the false negative results

NEW STRATEGIES: Recombinant proteins expressed in different expression Systems





Validation of antibody detection ELISA tests based on baculovirus-expressed recombinant p54, p32 pp62 and Dual or HT (p32-15) proteins antigens.



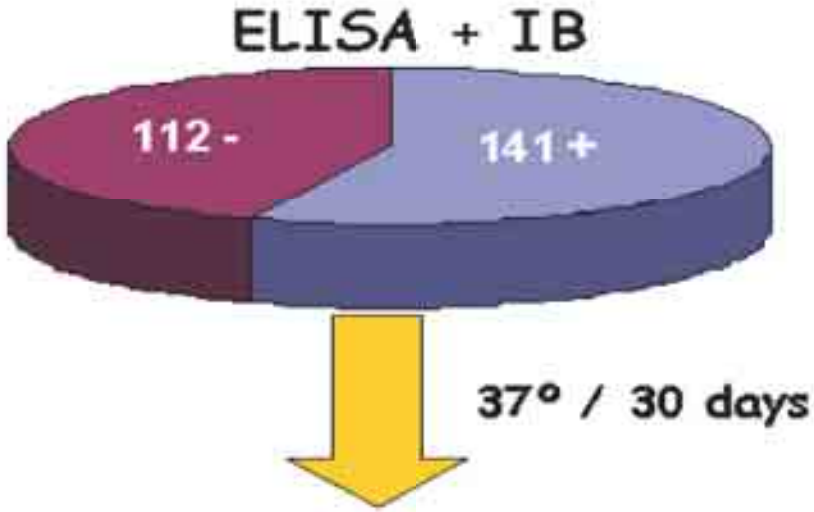
Collection of sera from Europe and west African countries (different years and origins).

	Sensitivity	Specificity
P32-ELISA	99.3	97.4
P54-ELISA	91.1	96.9
PP62-ELISA	99.6	98.2
OIE-ELISA	97,4	87.3
P32-15-ELISA	**98,2**	**99,3**

CONCLUSIONS

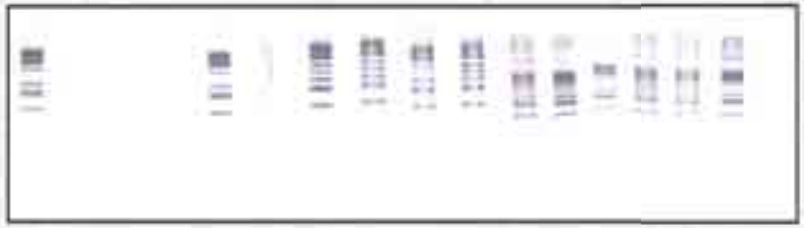
i) The use in ELISA of these recombinant proteins show **similar** or even better results in **sensitivity** and **improve the specificity** of the conventional ELISA.

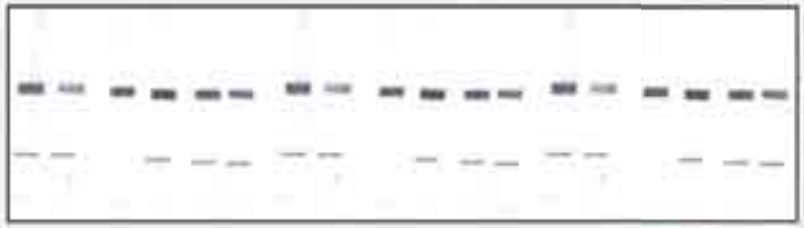
ASSESSMENT OF SUITABILITY OF THE NEW ASSAYS TO TEST SERA POORLY PRESERVED



	Sensitivity	Specificity
p32-ELISA	98.5	96.4
p54-ELISA	97.1	93.7
pp62-ELISA	100	100
Dual- ELISA	97.5	98.1
HT- ELISA	98.2	95.3
OIE-ELISA	80.8	92,8

Confirmation by Western Blot





Significant Improvement of the results with poorly preserved sera –resembling field situation in Africa and in Europe during summer season.

ASSESSMENT OF SUITABILITY OF THE NEW ASSAYS TO TEST EAST AFRICAN SERA

The percentages of specificity and sensitivity obtained analysing the Tanzania and Uganda field sera with the different recombinant ELISAs and OIE-ELISA are shown in these tables .From the results shown in these tables we can conclude that in the analysis of sera collected from East African countries with the rP-ELISAs we found a loss of sensitivity not identifying the total of positive sera by IB test and not improving the results obtained with the OIE-ELISA.

Domestic pigs

Tanzania sera (169)

A)	Sensitivity	Specificity
OIE-ELISA	67	97
p32-ELISA	33	90
p54-ELISA	0	100
Dual - ELISA	33	95
HT-ELISA	33	92

Uganda sera (189)

Domestic pigs and warthogs

B)	Sensitivity	Specificity
OIE-ELISA	67	94
p32-ELISA	70	93
p54-ELISA	30	98
Dual - ELISA	78	93
HT-ELISA	85	95

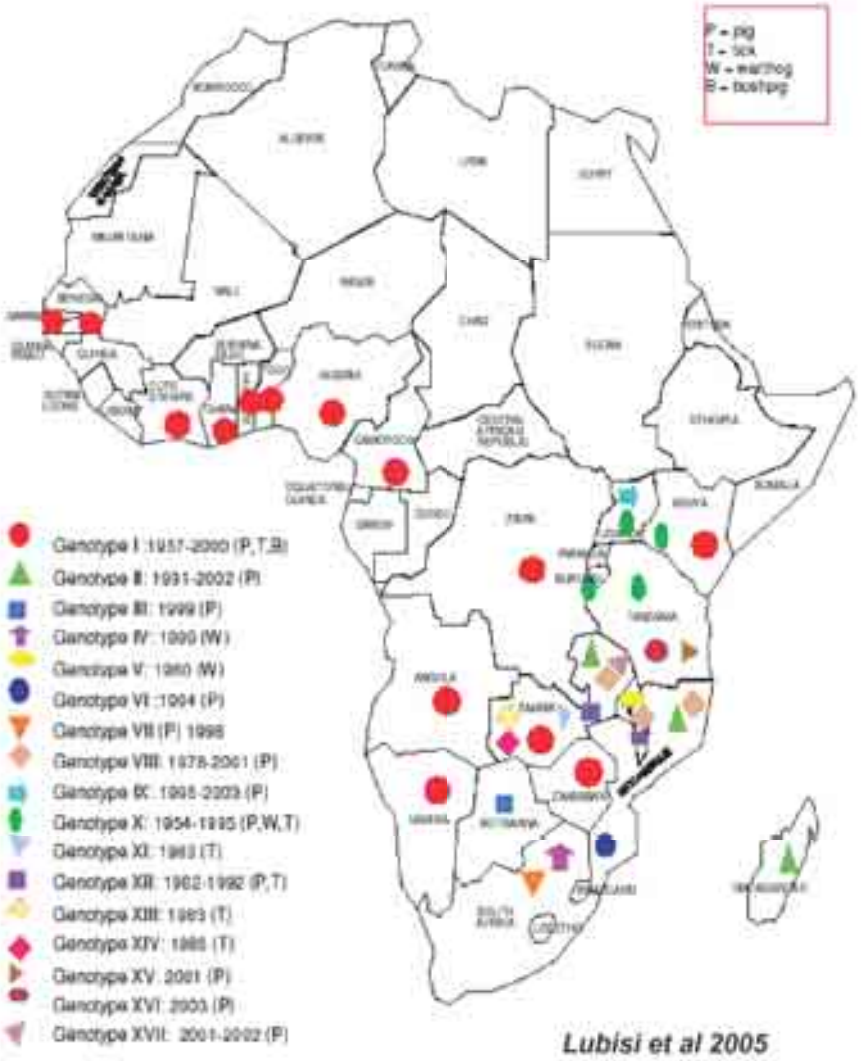
Are serological tests failing in sensitivity due to the variability of the new isolates circulating in eastern African countries??

MOLECULAR EPIDEMIOLOGY





Genotyping of ASFV isolates by partial p72 gene characterization



Lubisi et al 2005

p72 genotype I:

It includes a wide group of viruses from Europe, the Caribbean and West and Central Africa, obtained over a wide time period. - ex. Portugal 1959 and as recently as 2006 in West Africa or Sardinia. -

Recent studies also show high variability in p54 and significant in p32 (85-94%)compared with West african and European ASFV isolates.

To Date NO VACCINE AVAILABLE AGAINST ASF

Why?

The difficulty in inducing effective immunity may be related .:
-To the great variability observed among ASFV isolates.
-To the fact that ASFV replicates in some cells typically involved in the immune response.

- Causative agent of ASF is not an "unique" virus: We should talk about a "family" of virus (multigenic families).

-Little is known about immune response and immune protection mechanism . The role of the humoral and cell mediated immunity in protection are still poorly understood.
-Experimentally, recovered pigs produced normal levels of neutralising abs. against FMD.
- It has been shown ASF specific antibodies to delay the onset of the clinical signs, reducing levels of viremia and protect pigs against death.

PREVIOUS STRATEGIES based on:

NOT PROTECTION

Immunization with live attenuated vaccines

- Experimentally, some animals get protection against *homologous* strains of virus., but become carriers and develop chronic lessions.

Inactivated vaccines, and immunization with viral proteins p54, p30 and viral (HA)- homoloy to CD2.

- ✓ Ab against p73 and p54 neutralized viral attachment(*in vitro*)
- ✓ Ab against p30 inhibited virus internalization into cell (*in vitro*)
- ✓ The HA of ASFV might induce a strong T-cell response

Deletion Mutants.

CURRENT PLANNING PROGRAMME FOR ASF CONTROL IN SOME REGIONS OF AFRICA :

- To set up associations of producers on the scale of the village, sector, area,
- To create on the level of each village a local committee of defense
- To organize the stockbreeders,
- To set up a system of micro-credits.
- To set up good diagnostic test for a rapid detection
- To organize the detection of the virus carriers
- To eliminate the chronic carriers,
- To create a control structure under the control of the veterinary Services.:
  - To set up and to reinforce the sectoral committees for control animal movements and/or trade,
  - To set up a travel autorization system for the animals
  - Application of measures of seizure for the contraveners....

NEW RESEARCH

National and international (EU, Wellcome Trust, INIA, …) Funds

Future work ...

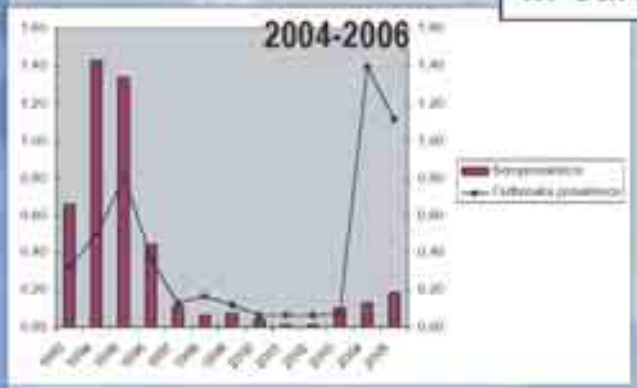
LATEST FINDINGS.:





OIE and mainly new rp- ELISA test show low sensitivity of Ab.detection

In Sardinia:



Relationship between outbreaks and seroprevalence.
Source: Annual meeting 2006

Serological screening: seems to be no relationship between the low seroprevalence of seropositive pigs and the high incidence of ASF in the region.

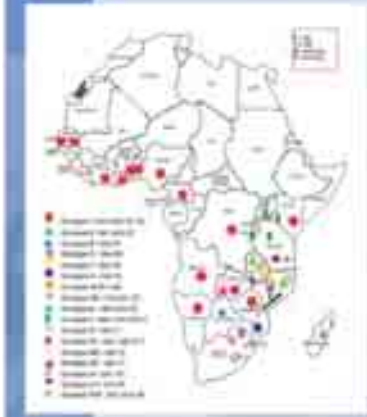
In eastern regions of Africa:

Similar pattern seems to be observed in several eastern regions: , **low seroprevalence and high percentage of virus- positive animals.**
In contrast , and at the same time in the recent outbreaks of ASF in neighboring regions the virus appears to be more virulent .

THIS DATA MAY SUGGEST:

- Presence of attenuated or non-virulent strains (**causing asymptomatic disease in domestic pigs, acting like reservoirs for ASF**) co-existing with virulent ASF isolates in the same region?
- Are serological tests failing in sensitivity due to the variability of the new isolates??

There is a need of evaluate the real extension of the changing and to deal with its possible consequences.



EPIDEMIOLOGY Further Studies:

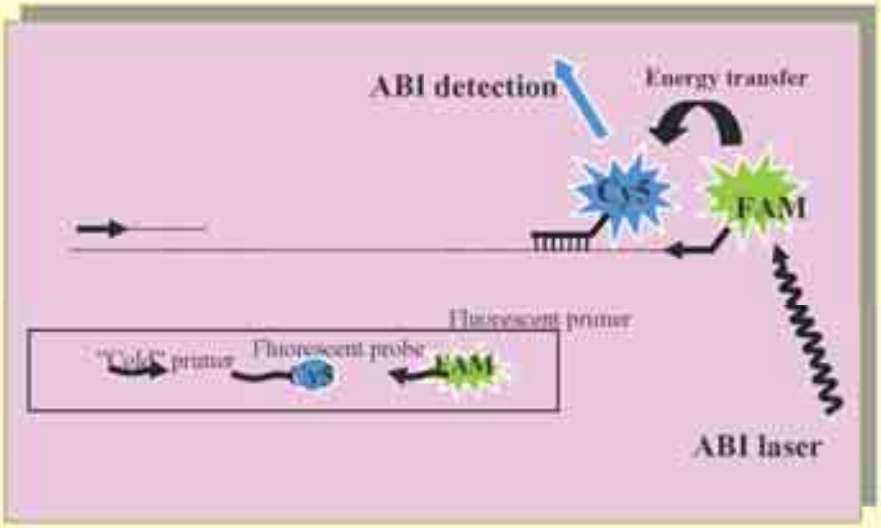
Increase the knowledge of New Circulating Isolates and their Molecular Features,

- Analysis of current epidemiological situation of ASF in Africa and Sardinia.
- Molecular and biological characterization of current circulating field viruses in Africa and Sardinia.
- Risk analysis of the introduction of ASFV in the EU countries
- Development of new control strategies focused on the situation in Africa.

VIRAL DETECTION TECHNIQUES.

Future reseach

- **New Real-time PCR assays** (New Taq-man and a PriProET PCRs) and to Adapt PCR systems to commercial portable PCR machines for on-site diagnosis.
- Development of **Isothermal Amplification assays** for rapid detection of ASFV. Adaptation to on site diagnosis by a simple, inexpensive and portable platform.
- **DNA purification techniques**, using FTA and 3MM filters. Application to on site diagnosis



PriProET PCR systems

ANTIBODY DETECTION TECHNIQUES.
Future reseach

-**New ELISA tests based on antigens** (new recombinant proteins –p72,pp62,p54, p32 and identified new candidates,) obtained from the current circulating virus isolates from Eastern southern Africa and Sardinia. **New IB tests.**

-**New ELISA tests based on the use of a soluble cytoplasmic ASFV antigen obtained from African isolates.**

- **ELISA tests based on the determination of specific IgM antibodies**

-**Penside test and front line diagnostic tests for antibody detection using a dip-stick format (dot-blot assays) with the new antigens.**

To be used for serological surveillance and control of ASF in endemic areas





HOST-VIRUS INTERATIONS AND VIRAL IMMUNE RESPONSE:
Future research

- **ROLE OF VIRUS AND HOST GENES IN INFECTION:** virus genes and cellular factors involved in the control of host defences, immune evasion and virus productivity
- CHARACTERIZATION OF ASFV VIRULENCE FACTORS.
- HOST RESPONSE TO INFECTION
- DEVELOPMENT OF ANTIVIRAL MOLECULES.

CURRENT STRATEGIES FOR A VACCINE:

- VACCINATION WITH DNA VACCINES.- Up to date without successful results.
- IMMUNIZATION WITH NEW RECOMBINANT PROTEINS CANDIDATES.
- VACCINATION WITH DELETION MUTANTS.

PREVENTION

•An important source of ASF entrance in free areas is by contaminated garbage from international airports and ports.

All food leftovers from planes and ships should be incinerated

•In endemic areas, the control of movements and extensive serological surveys to detect the carrier pigs is one of the most important aspect of prevention to avoid new infected regions.

•In endemic areas of Africa, the control of natural reservoir, soft ticks and warthogs, and preventing their contact with domestic pigs

•When ASF is suspected, pig movements should be restricted and diagnostic test should be performed immediately

LOW VIRULENCE ASF STRAINS DO NOT CAUSE SIGNS OR LESIONS THAT SIGNAL THEIR PRESENCE

African Swine Fever

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THANKS To You for your Atention