RESEARCH REPORT
SWINE HEALTH

**Title:** Development of fluorescent recombinant antibodies to detect African swine fever virus in tissue samples and infected cells. **NPB #11-022**

**Investigator:** Dr. José M. Escribano

**Institution:** Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) Spain

**Date Submitted:** February 13, 2013

**Industry Summary:**

The present project pretends to develop new reagents to solve an important gap in the African swine fever virus (ASFV) diagnosis. This virus is nowadays a real threat for Europe and, potentially, may spread to Asia or even other continents where the virus may produce a tremendous impact in the pig production.

The serology of ASF has been resolved by the use of recombinant proteins in the diagnostic tests. These proteins have been validated and represent a better alternative to antigens obtained from infected cells (use of infectious virus for their generation). Recombinant antigens showed an improved sensitivity and specificity for antibody detection in chronically infected or innaparent carrier pigs and allowed the standardization of reagents production and tests interpretation.

However, for the control or eradication of this important swine disease (no vaccine is available) is necessary to combine serology surveys with techniques for virus detection in samples of potentially infected pigs. Rapid detection means a minimization of disease spread risks to other animals or farms. Actually, the virus detection has to be done by PCR analysis (detection of viral DNA). This methodology detects accurately the virus presence in pig tissues, but needs a reference confirmatory technique because frequent false positive results, specially in laboratories with a reduced training level. The OIE recommend the virus isolation and the virus detection in animal

tissues by antibody immunofluorescence. There are not available commercial universal reagents (antibodies) to carry out the immunofluorescence tests on tissue cuts or tissue explants. Additionally, the virus has to be isolated in primary pig macrophage cultures and, frequently, it takes several days and even weeks, depending of the virus titers in body fluids or organs, before the observation of the characteristic cytopathic effect or the haemadsorption reaction.

The main objective of this project was to develop recombinant antibodies that could be used as reagent for sensitive detection of the virus in biological samples or infected cell cultures used for virus isolation. These antibodies, labeled with fluorescent molecules, will allow the virus detection using different technologies. These recombinant antibodies will avoid the use of sera from infected animals (potential risk of virus contaminations) or the use of monoclonal antibodies directed to variable epitopes of the virus that could fail in the detection of any specific virus strain. Antibodies would be produced by a cost-efficient system based on baculovirus vectors (a common system to produce biologics) and insect larva (living biofactories) instead insect cells. The larva system, only used for the moment by a reduced number of companies and research laboratories, is one of the most efficient and cost-effective system to produce any recombinant protein. These reagents (recombinant labeled antibodies) could be sent, without any risk, to reference diagnostic laboratories and would facilitate the standardization of results, independently of the expertise of profesionals in ASF diagnosis. The limitation of the source of these antibodies would not be a problem for diagnostic laboratories in contrast to the limited source of antibodies obtained from immunized or naturally infected pigs.

The conducted research during the granted 1 year project has generated different recombinant antibodies which are able to accurately detect ASFV in cell cultures. These antibodies were generated in insect larvae (IBES technology) with excellent productivities. Those useful reagents will be tested during the next months in samples from experimentally infected pigs to certify their sensitivity in virus detection and will also be tested in diagnostic laboratories from endemic regions (South Africa and Russia). Once those reagents were validated, those will be transferred to a company for their commercialization in any potentially affected country, including USA. Results obtained will also be published during the next months to disseminate the scientific information to the veterinary scientific community.

Principal investigator: Dr. José M. Escribano
Departamento de Biotecnología. INIA. Spain
escriban@inia.es

**Keywords:**

African swine fever virus, diagnostic reagent, virus detection, antibody immunofluorescence, recombinant antibody

**Scientific Abstract:**

The present project pretends to develop new reagents to solve a gap in the ASFV diagnosis. The serology of ASF has been resolved by the use of recombinant proteins for serological tests (ELISA and confirmatory Immunoblotting). These proteins have been validated and represent a better alternative to antigens obtained from infected cells (use of infectious materials for their generation). Recombinant antigens showed an improved sensitivity and specificity for antibody detection in chronically infected or innaparent carrier pigs.

However, the virus detection has to be done by PCR analysis. This methodology detects accurately the virus presence in pig tissues, but needs a reference technique. The OIE recommend the virus isolation and the virus detection in animal tissues by immunofluorescence. There are not available commercial universal reagents (antibodies) to carry out the immunofluorescence tests on tissue cuts or tissue explants. Additionally, the virus has to be isolated in primary pig macrophage cultures and, frequently, it takes several days and even weeks, depending of the virus titers in body fluids or organs, before the observation of the characteristic cytopathic effect or the haemadsorption reaction. The virus detection in infected cultures can be accelerated by the use of fluorescent antibodies directed against structural antigenically conserved virus proteins. The main objective of this project is to develop recombinant antibodies that could be used as a reagent for sensitive detection of the virus in biological samples. These antibodies will be labeled with fluorescent molecules that allow the virus detection using different technologies. These recombinant antibodies will avoid the use of sera from infected animals (potential risk of virus contaminations) or the use of monoclonal antibodies directed to variable epitopes of the virus that could fail in the detection of any specific virus strain. Antibodies would be produced by a cost-efficient system based on baculovirus vectors and insect larva (living biofactories), which is one of the most efficient system to produce recombinant proteins. These reagents (recombinant labeled antibodies) could be sent without any risk to reference laboratories and would facilitate the standardization of results independently of the expertise of technicians in ASF diagnosis. The limitation of the source of these antibodies would not be a problem in contrast to antibodies obtained from immunized or naturally infected pigs.

Two approaches have been carried out to cover the objectives of the project. The first consists on the generation of a cDNA antibody library from the spleen cells of mice immunized with the recombinant ASFV protein p54. Several recombinant single-chain antibodies were produced in the

baculovirus expression system and used for the ASFV detection in cell cultures. The second approach consisted in the generation of a cDNA single domain (camelid antibodies) antibody library from llama immunized with the same recombinant protein p54. The screening of the library will render highly reacting antibody clones which will be subcloned and expressed in the baculovirus expression system (work in process). To advance about the feasibility of using baculovirus-derived fluorescent single domain antibodies in diagnostic, we have modeled two antibodies of this nature but directed to a protein from Rotavirus A (specific for the VP6 protein). These antibodies were expressed fussed or not to the green fluorescent protein and used for the VP6 detection in cell cultures infected by a baculovirus expressing this protein. Production of recombinant fluorescent antibodies by IBES® technology was excellent as well as their functionality in VP6 detection by ELISA and immunofluorescence. Similar experiments will be carried out in the next months with the antibodies directed to the ASFV protein p54. With the approved extension of the present project, we will finish all objectives contemplated in the original proposal. We hope that the obtained results will be published during the first quarter of the next year. A final update of results will be send to the National Pork Board describing the final conclusions of our results before the end of next March 2013.

**Introduction:**

African swine fever virus (ASFV) infection has been well established over many years in a sylvatic cycle in East and southern Africa involving transmission between warthogs and the soft tick vector *Ornithodoros moubata*. The virus causes inapparent infections in these hosts (and in bushpigs) which can remain persistently infected over several years. African swine fever (ASF) was first reported in domestic pigs in E. Africa early in the 1900s when these came into contact with infected warthogs. Since then, ASF has been reported in most sub-Saharan African countries. In 1957 the first trans-continental spread of the virus occurred to Portugal and ASF remained endemic in Spain and Portugal from 1960 until the 1990s. Further transcontinental spread of disease occurred during the 1970s and 80's to South America and the Caribbean probably due to movement of infected pork in which virus survives for long periods. The most recent outbreaks of ASFV outside of Africa started at the beginning of 2007 in Georgia and from this country the virus spread to the neighbouring countries of Armenia, Azerbaijan and Russia. Although recent reports seem to indicate that China has been affected, the disease in this country has not officially declared. The continuous outbreaks outside Africa pointed out the risk of virus introduction in any country outside Africa. Once the disease is introduced in a specific country, the movement of animals or pig-derived products inside the affected country or to other countries is drastically restricted with a huge economic impact for pig producers.

Virus can be transmitted from wildlife to pigs by bites from infected ticks. In some African countries including Malawi, Mozambique, Zambia and Angola, ASF has become established as an enzootic disease in domestic pigs and is maintained in the absence of contact with warthogs. Although the original descriptions of ASF were of an acute haemorrhagic fever causing mortality approaching 100%, less virulent isolates have emerged as disease has circulated in domestic pigs. Pigs which recover from infection can remain persistently infected and excrete virus over long periods thus providing a reservoir for infecting healthy pigs. Increasing numbers of low virulence isolates were reported in Spain and Portugal during the 1970s and 80s, some of which caused inapparent infections. Although the mechanisms by which ASFV is maintained in domestic pig populations are often unknown the most likely risk factors are the movement of infected pigs and pork products.

There is no vaccine available against ASFV and disease control relies on rapid diagnosis and implementation of sanitary measures and movement restrictions. Diagnosis is complicated by the varying pathogenesis caused by different isolates, the similarity with other haemorrhagic fevers and the need to transport samples to labs for confirmatory tests. Implementing these control measures requires a good infrastructure, availability of reagents and expertise which is lacking in many countries. The rapid screening and diagnostic of suspicious infected animals is very important to avoid rapid dissemination of the disease. In USA only a few laboratories have the appropriate technologies and reagents for ASFV diagnostic and no kits for ASFV diagnostic are commercialized in North America. One of the reasons is because specific antibodies and antigens have to be produced with infectious virus and have to be done under high level of containment (BSL3 facilities). Only recently, recombinant antigens have been developed for serodiagnosis of the disease in subacute infections and innaparent carrier pigs. Only one company (Ingenasa. Spain) commercializes in Europe monoclonal antibodies against this virus and they have not been tested against many circulating viruses. Then, there is an urgent necessity for reagents that allow virus and specific antibodies detection.

The continuous existing risk of ASFV introduction in any country, as recently demonstrated in Russia and nearby countries, constitute a factor to be considered seriously by pig producers in USA. It makes extremely important to be prepared for the rapid diagnostic and eradication of the disease early after the first outbreaks. The rapid detection of ASF, as in other diseases, definitively determines the spread of the virus, the reduction of the number of affected farms and the possibilities to avoid the apparition of ASF subacute cases more difficult to detect. The availability of safe and diagnostic efficient recombinant DNA-produced reagents will allow to the U.S. animal

health authorities the dissemination of reagents to diagnostic labs and, in case of need, the unlimited source of diagnostic tests to control ASF in absence of an available vaccine. The continuous movement of people and goods among different continents increase the risk of ASFV introduction in U.S. Because ASF is a disease of economically poor countries, developed countries have to be implicated in the development of eradication tools for this important disease, even if they are not affected for the moment.

**Objectives:**

The main objective of this project is simply but relevant in ASFV diagnosis. We propose the development of new antibody-based tools for ASFV detection in pig macrophages in cell cultures and tissue samples from infected pigs. To reach this objective, we proposed the development of recombinant antibodies (mouse and camelid single domains) fused to fluorescent proteins (red and/or green). Those antibodies will be produced by recombinant baculoviruses in insects (lepidopter *Trichoplusia ni*) as living biofactories (IBES® technology) for efficient scaling up production and for reducing the production costs. Antibodies would be used directly over cells *in vitro* (used for virus isolation), tissue cuts or tissue explants for virus detection. Those reagents would be universal reagents to facilitate the virus detection by a simple technique (antibody immunofluorescence), providing a diagnostic test of easy interpretation for non expert diagnosticians in ASF.

**Materials & Methods:**

Among all ASFV proteins, the structural p54 antigen is one of the most abundant and antigenic essential virus proteins and is highly stable due to its relevant function during infection. This protein has been used for serological diagnosis of the virus and has been produced in a recombinant way by different technologies by our group. Additionally, protein p54 accumulates at high level in infected cells facilitating its detection by antibodies. Then, protein p54 containing a 6xHis tag and expressed in *E. coli* was purified by $Co^{2+}$-based Immobilized Metal Affinity Chromatography (IMAC) resins (TALON®, Clontech, USA) and used to immunize mice and llamas to obtain plasma cells producing specific antibodies against this protein.

**Generation of DNA p54-specific antibody libraries from mice plasma cells-** For the development of the project, we constructed an antibody library in phages of spleens from immunized mice with the purified p54 (3 antigen doses of 30µg). Extraction of total RNA from the spleens was done with Trizol (Invitrogen) followed by the method (Qiagen) guanidinium thiocyanate. The synthesis of cDNA

from RNA was carried out with oligo dT and polymerase SuperscriptIII (Invitrogen) followed by PCR using a collection of specific mouse oligonucleotides that cover the repertoire of immunoglobulins. PCR reactions were carried out according to standard protocols with 30 cycles. VL and VH PCR products were purified using appropriate resins (Qiagen) and were combined using SOE-PCR using oligonucleotides coding for the spacer sequence between the two domains (Gly4Ser) x 3, overlaping each other and enabling the extension and union of PCR fragments. Overlaping PCR fragments were subcloned in the phagemid vector pIT2, and used for electroporation of the *E. coli* strains XL1blue or TG1, which were ampicillin plaqued on 530 cm2 plates. The obtained colonies were grown in 8 ml of 2xYT media and frozen in glycerol at - 80 ° C. The rescue of fagemid particles was done with cooperating phage KM13, containing the gene for resistance to kanamycin. The Phage library prepared was ready for selection or later amplification.

For the selection, they were used between $10^{12}$ and $10^{13}$ PFUs. These phages were used to immune react with p54 antigen in ELISA (Falcon, BD) 96-well plates. Subsequent rounds of selection with decreasing amounts of antigen, were used to improve affinity antibody selection. Once a population of phages gave a clear difference between positive and negative antigens, we selected individual clones. The expression of the fragment antibody was induced with 1 mM IPTG for 16 hours at 30 ° C. Since the functional scFv are in the periplasmic region, several rounds of bacteria freezing and thawing were done to release the content of the periplasm. The recognition by the scFvs of the antigen was checked by ELISA, with a secondary monoclonal antibody against peptide c-myc which is fused on genetic constructs to the antibody fragments. ScFvs giving positive signal in ELISA were sequenced and analyzed by PCR amplification with specific primers in order to establish its diversity.

**Generation of DNA p54-specific single domain antibody libraries from llama-** The production of recombinant llama-derived VHHs against protein p54 was contracted to Inmunova company, Buenos Aires, Argentina. The protocol used by the company is summarized as follows:

**Llama immunization.** A 1-year-old male llama received five doses of 100µg of purified ASFV p54 protein, emulsified in oil adjuvant (42.5% Marcol 52, 6.5% Arlacel C, 1% Tween 80), at days 0, 21, 28, 35, and 90. Serum and blood samples were taken at days 0, 4, and 7 after each inoculation. The antibody response was monitored by ELISA. To evaluate the effector B-cell response, an ELISPOT assay determining the number of p54-specific antibody-secreting cells (ASC) in the peripheral blood of the inoculated llama was adapted.

**VHH library production and enrichment of p54 binders.** From a total of 900 ml of blood collected 4 days after the last injection, 6-$10^8$ mononuclear cells were extracted by Ficoll-Paque gradient centrifugation, pelleted, frozen in liquid nitrogen, and then kept at -80°C. The total RNA was extracted by using an RNA extraction kit (Nucleospin RNA II; Macherey Nagel), yielding 256 µg of RNA. Subsequently, first-strand cDNA was synthesized from 210µg of RNA by using Superscript III reverse transcriptase (Invitrogen), with oligo(dT) primers (Invitrogen) or random primers (Invitrogen). In a 20µl reaction mixture 0.2, 1, or 5µg of total RNA were used. The cDNA encoding VHH and VH was specifically amplified by PCR using the primers CALL01 and CALL02 annealing at the leader and at the CH2 sequences. The 600-bp fragment (VHH-CH2 without the CH1 exon) was eluted from a 1.6% agarose gel after separation from the 900-bp fragment (VH-CH1-CH2 exons). VHH fragments were then amplified with one additional nested PCR with primers annealing at the framework 1 and framework 4 regions, followed by the use of primers containing the restriction sites for further cloning steps: VHHfor2 containing the NcoI and PstI restriction sites, and VHHrev2 containing the NotI restriction site. The final PCR fragments were ligated by using the upstream restriction sites NcoI or PstI and the downstream NotI site into the phagemid vector pAO-Lib, carrying a long irrelevant sequence that is removed upon VHH insertion in order to slow down the potential propagation of vector without a VHH insert. Ligated material was transformed into *E.coli* TG1 cells. The colonies from the plated cells were collected, washed, and stored at -80°C in LB medium supplemented with glycerol (50% final concentration). Specific VHHs were selected from the library using the phage display technology, similarly as described for the antibody libraries obtained from mice. After the second or third round of biopanning, individual colonies were grown, and the corresponding VHH clones were analyzed by phage ELISA and sequenced for diversity.

**Screening for p54 specific VHH fragments (phage ELISA).** Phages displaying the selected VHHs were produced by the individual TG1 *E. coli* clones. Produced phages were tested by ELISA using recombinant p54. An antigen produced also in *E. coli* was used as negative control antigen. After the coating step, plates were blocked with 4% skim milk in 0.5% Tween 20–PBS. Phages were added, followed by incubation at room temperature for 60 min. The assays were developed using a 1/5,000 dilution of a horseradish peroxidase–anti-M13p8 conjugate (Amersham/ Pharmacia Biotech) for 40 min at room temperature, followed by H2O2/ABTS [2,2_azinobis(3-ethylbenzthiazolinesulfonic acid)] as a substrate chromogen reagent.

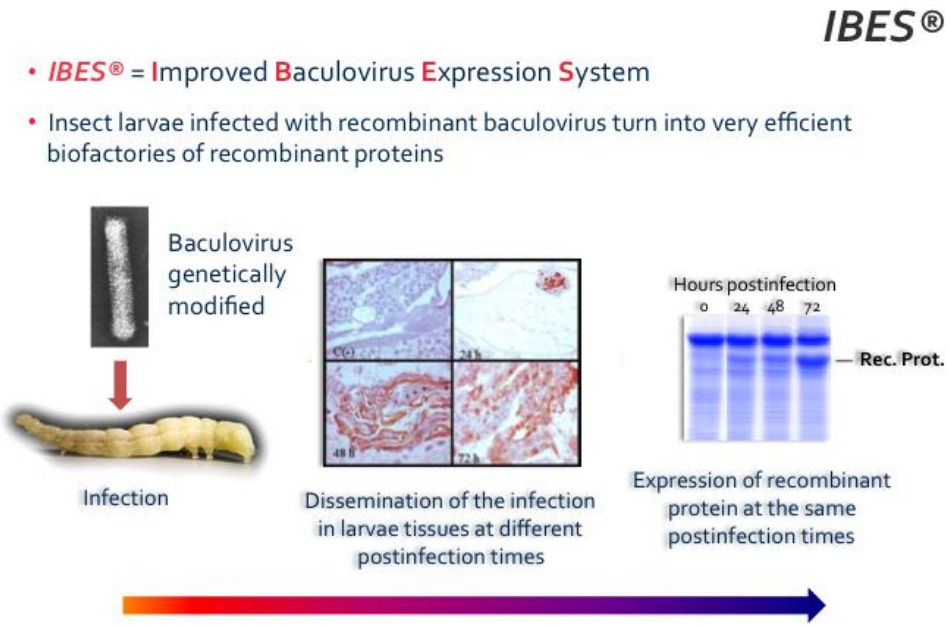
**Generation of recombinant baculoviruses expressing recombinant single chain and single domain antibodies fused to a green fluorescent protein.-** There are currently a large number of fluorescent proteins in the market. These cover a wide range of colors that pose an additional advantage in selecting the most appropriate in the development of new diagnostic technologies, or

combine them. Employment of antibodies marked with fluorescent proteins can open a new line inside the diagnosis.

The sequence coding for a scFv or a VHH specific for p54 protein with higher protein affinity obtained from mice and llama respectively were amplify by PCR using oligos specially designed to clone the genes into a pFastBac vector which contains fused at the 3′ end of the gene (c-terminus) the gene coding for the EGFP protein (green). The baculoviruses were generated through the Invitrogen "Bac-to-Bac Baculovirus expression System. All cloned antibodies contained the insect signal sequence derived from the honeybee melitin to facilitate the protein processing after synthesis. Recombinant baculoviruses were used to infect insect cells in culture (Sf21) and after 72 hours, we observed the antibody expression by fluorescence. Viruses were passed for three times in insect cells to reach virus titers of about $10^8$ PFUs/ml

**Expression of recombinant fluorescent antibodies in insect larvae.-** The improved baculovirus expression system (IBES) technology, is the most advantageous and efficient baculovirus-based technology to produce any kind of functional recombinant protein by the use of insects as small living biofactories. IBES technology can be used to produce vaccines, diagnostics, therapeutic molecules and other protein-based products.

*Trichoplusia ni* (*T. ni*, Cabbage looper) larvae were reared under confined conditions. For all experiments, fifth-instar larvae will be injected with the recombinant baculoviruses near the proleg (forward the body cavity) using 10µl of different plaque forming units (PFUs)/larva doses, to establish the optimal conditions of antibody productivity in larva. At different post-infection times, larvae were collected, immediately frozen and kept at -20ºC until processed. Recombinant antibodies were used with a simple clarification of the larvae-derived soluble protein fraction.




**Establishment of the optimal conditions for the use of recombinant antibodies in infected cell cultures.-** To determine the recombinant antibody reactivity with protein p54, insect cells or Vero cells were infected by recombinant baculoviruses or ASFV and after 24-72 h post infection were used to react with larva-derived antibodies. Permeabilized cells were incubated with different recombinant antibody concentrations and observed by immunofluorescence or confocal microscopy to see cellular distribution of fluorescence.

**Results:**

**- Development of a reference serum:**

To develop a gold standard reagent which allow us to compare results obtained with the recombinant antibodies that will be developed in the present project to be used in virus detection by immunofluorescence, the highly antigenically preserved recombinant protein p54 was produced in *E. coli* and used to immunize a pig. The animal was repetitively immunized with 100 micrograms doses of the purified protein to reach antibody titers higher than 1:40,000 by ELISA. The animal was bled and the serum obtained was aliquot and tested for virus detection in infected Vero cells at different post-infection times to determine the optimal dilution to be used in diagnosis. The serum detected accurately the virus protein p54 as early as 12 h post-infection, showing characteristic images of perinuclear virus replication bodies in infected cells.

- **Generation of DNA p54-specific antibody libraries from mice plasma cells**

Three mice were immunized intraperitoneally with four doses of 30 micrograms of purified ASFV protein p54 produced in *E- coli*. Mice showed specific antibody titers higher than 1:20,000 by ELISA 30 days after the last immunization dose. Mice were euthanized and spleens were mixed and processed to obtain a phage antibody library.

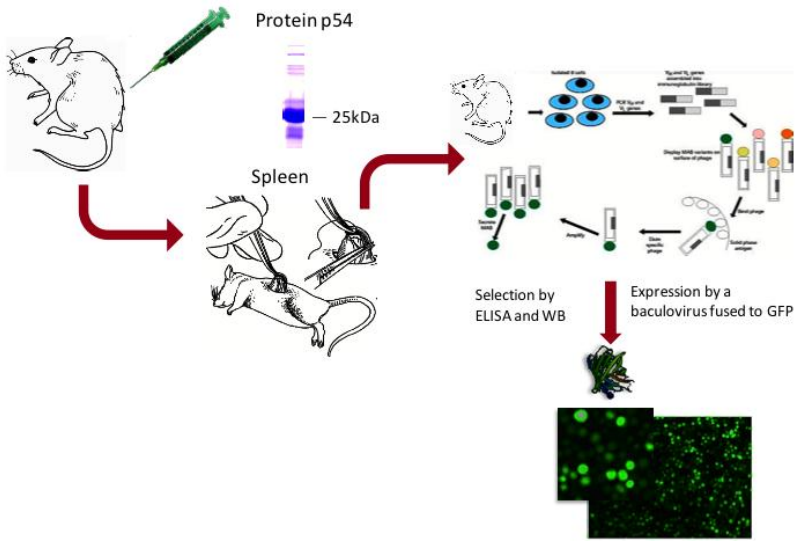



***Figure 1:*** *Schematic representation of procedures to obtain recombinant antibodies from cDNAs obtained from spleens of immunized mice with the ASFV protein p54*

Four single-chain antibodies specific for p54 protein were selected by their reactivity in ELISA and Western blot. Those antibodies were subcloned in a baculovirus expression system and expressed fused to the GFP protein. Recombinant antibodies were expressed in insect larvae (IBES® technology). Recombinant molecules were efficiently expressed as observed in figure 2. The recombinant antibody was expressed in larvae at higher levels than found in insect cells in culture (data not shown).

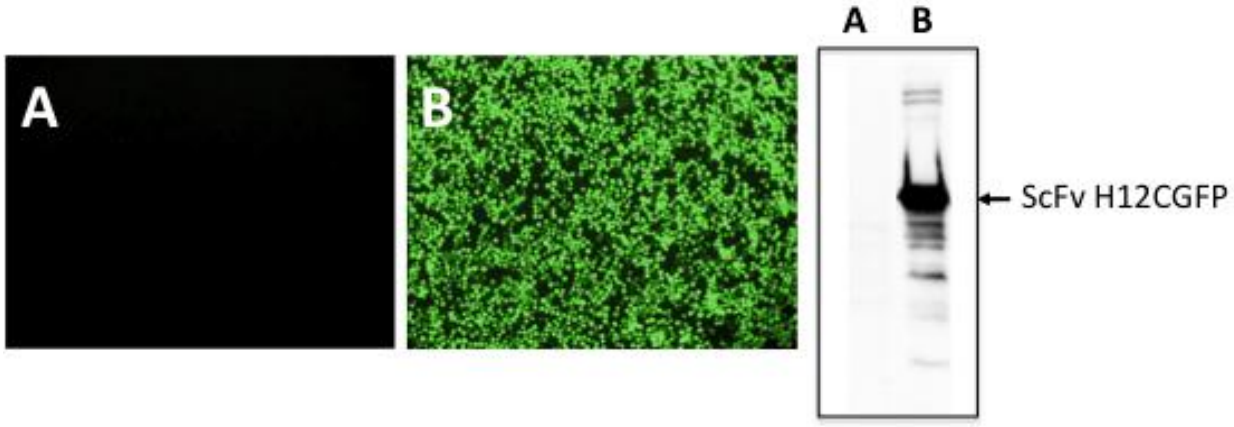



***Figure 2:*** *Expression in insect Sf21 cells of a single-chain antibody (ScFv) against protein p54 (H12C) fused to the green fluorescent protein (GFP). Panel A: mock infected cells; Panel B: insect cells infected with the baculovirus expressing the ScFv H12CGFP chimeric protein; This figure shows also the Western blot with an anti-GFP protein antibody of soluble protein extracts from mock- or baculovirus-infected insect larvae expressing the chimeric fluorescent antibody. The molecular weight of the recombinant protein was as predicted.*

From all fluorescent single-chain antibodies tested, only one antibody (H12C) detected the protein p54 in a immunofluorescence assay using permeabilized insect cells infected by a baculovirus expressing the protein p54 fused to the red fluorescent protein.

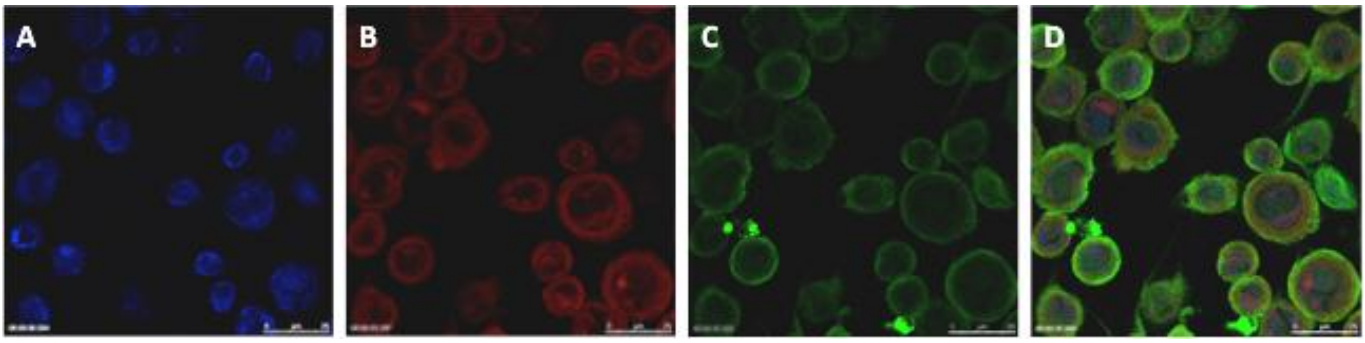



***Figure 3:*** *Detection of protein p54 by recombinant fluorescent single-chain antibody H12CGFP in insect SF21 cells infected with a baculovirus vector expressing the recombinant protein p54 fused to the cherry fluorescent protein (CFP). Panel A: Hoecht staining of insect cells; Panel B: Red fluorescence of insect cells infected with the baculovirus expressing the protein p54CFP (48h post-infection at a MOI of 1); Panel C: the same cells incubated with the recombinant antibody ScFv H12CGFP; Panel D: Merge image of red and green fluorescence in the confocal microscope.*

Once it was demonstrated that the recombinant antibody ScFv H12CGFP is able to detect the protein p54 by antibody immunofluorescence, we tested this antibody in ASFV-infected Vero cells. Infected Vero cells (72h post-infection at a MOI of 0.1) were permeabilized and incubated with a insect-derived non-purified protein extract containing recombinant antibody ScFv H12CGFP. The recombinant fluorescent antibody was able to detect accurately the infected cells, in a similar way than a monoclonal antibody directed to the capsid virus protein p72 (Ingenasa, Spain).

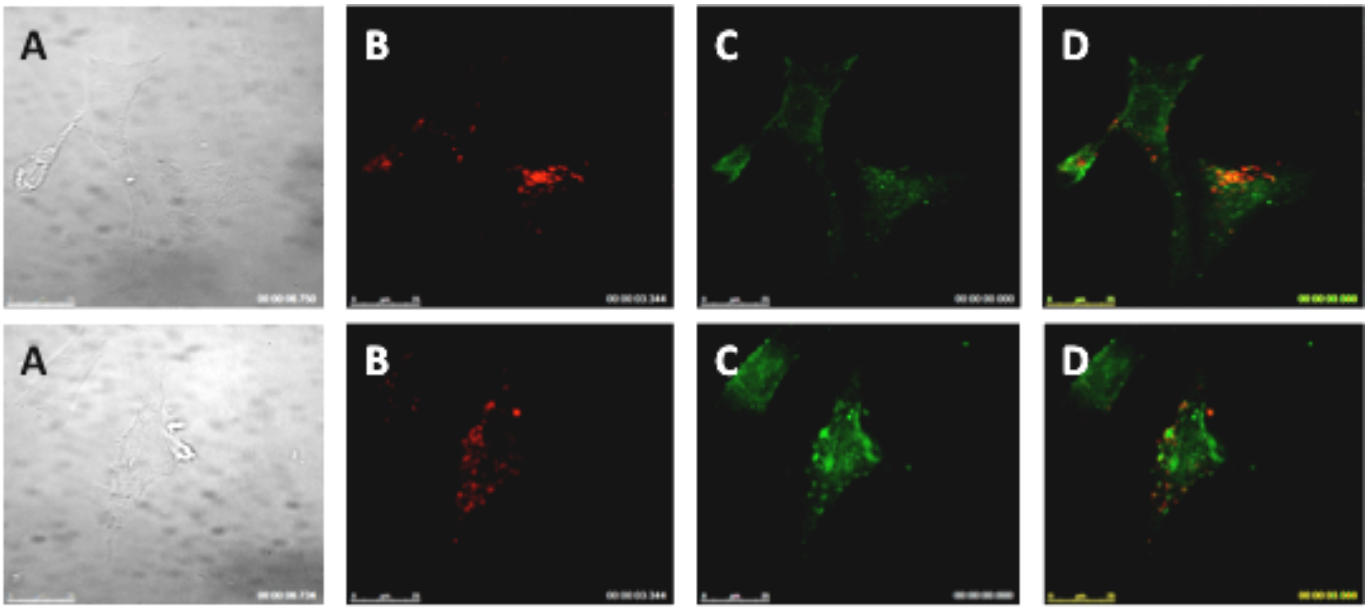



***Figure 4:*** *ASFV detection in Vero cells infected during 72h at a MOI of 1. Panel A: contrast microscopy of cell cultures; Panel B: Infected Vero cells incubated with a monoclonal antibody against the ASFV capsid protein p72. Panel C: The same cells incubated with recombinant fluorescent antibody ScFv H12CGFP; Panel D: Merge image of the two immunofluorescences obtained with both antibodies (B and C).*

**- Generation of DNA p54-specific single domain antibody libraries from llama**

Purified recombinant protein p54 was sent to the company Inmunova (Buenos Aires, Argentina) to generate the single domain libraries from llama. Animals were immunized (5 doses of 100µg each in oil adjuvant) and presented at the end of immunization process titers higher than 1:12,000 in ELISA. Antibody libraries were generated and screened with p54. A selected recombinant antibody cDNA was received at INIA and subcloned in the appropriate plasmids to generate recombinant baculoviruses. The antibody was expressed fused to the green fluorescent protein. The baculovirus obtained was titrated and used to infect insect larvae in order to produce the recombinant antibody by IBES® technology.

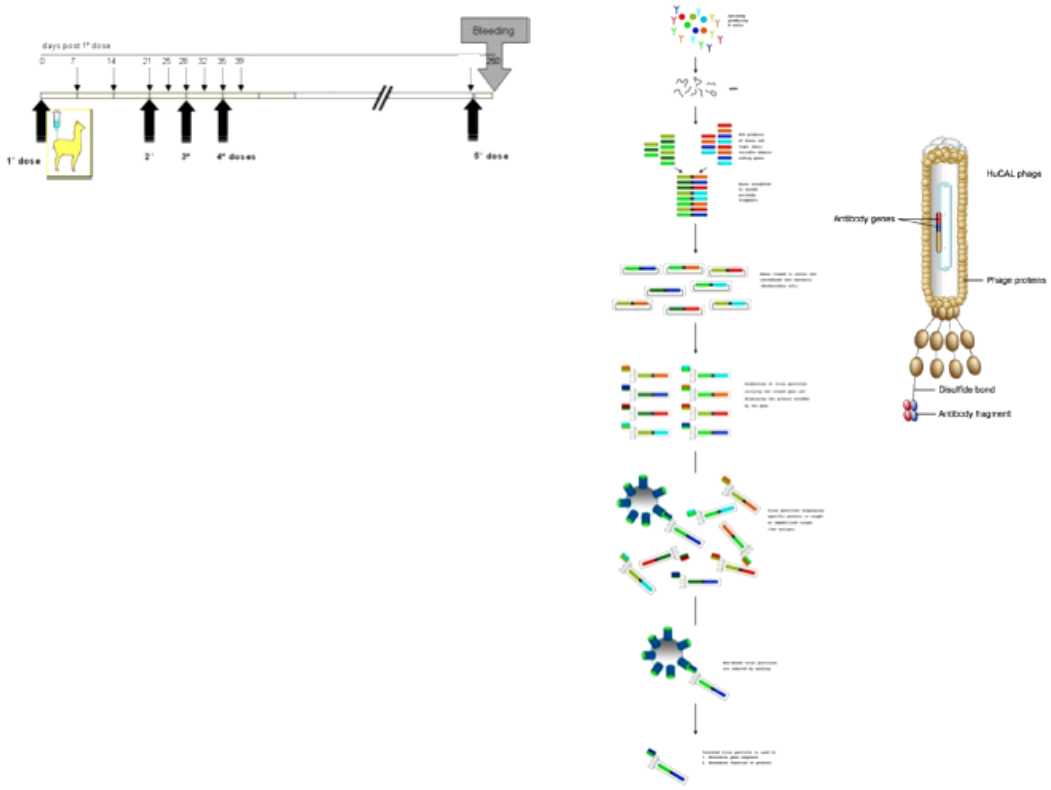



***Figure 5:*** *Schematic representation of procedures to obtain recombinant antibodies from cDNAs obtained from peripheral blood plasma cells of immunized llama with the ASFV protein p54*

To model the use of single domain antibodies in virus detection by immunofluorescence until receiving the anti-p54 recombinant antibody clones, two single domain antibodies against rotavirus A, previously obtained by our group, were cloned fused to GFP in baculovirus vectors. Both antibodies were expressed very efficiently in insect larvae by their respective recombinant baculovirus (figure 6).

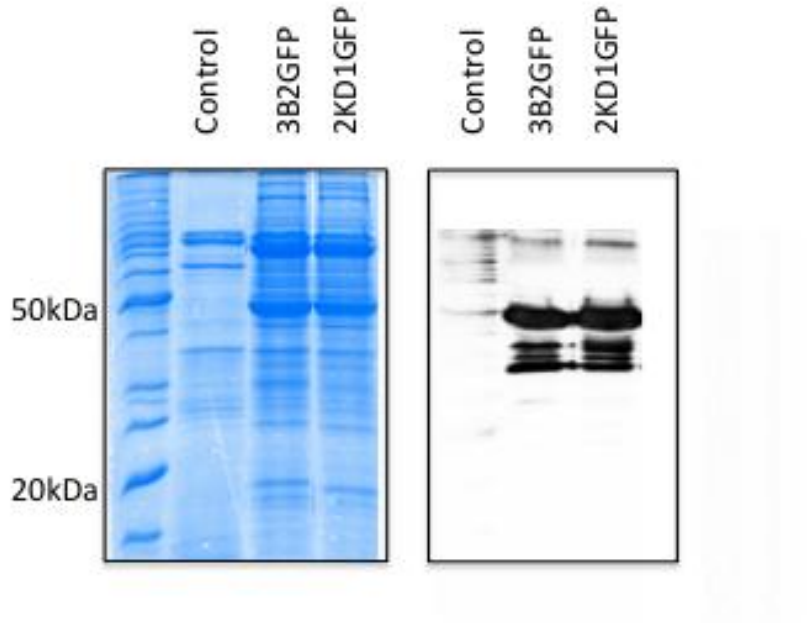



***Figure 6:*** *Expression of single domain antibodies 3B2 and 2KD1 fused to GFP. This figure shows the Coomassie blue staining of larvae extracts resolved by a SDS-PAGE and a Western blot with anti-GFP protein of the same extracts.*

Experiments with these recombinant antibodies (clarified crude soluble protein extracts from infected insect larvae) demonstrated that recombinant fluorescent antibodies were able to recognize the rotavirus A protein VP6. Permeabilized insect cells infected with a baculovirus expressing the VP6 (48h after infection at a MOI of 1) were incubated with the larva-derived fluorescent antibodies. Infected cells were successfully stained, demonstrating that VHHs fused to GFP can be used in immunofluorescence detection of viruses in biological samples.

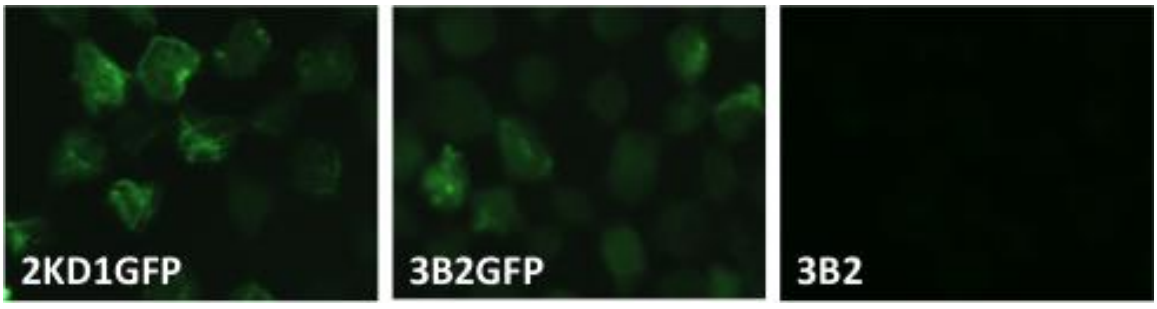



**Figure 7:** *Detection of the rotavirus A protein VP6 by recombinant fluorescent single-domain (VHH) antibodies 3B2 and 2KD1 in insect SF21 cells infected with a baculovirus vector expressing the recombinant protein VP6. Antibody 3B2 non-fused to GFP and expressed in the same system did not show any specific immunofluorescence in insect cells infected by the same recombinant baculovirus expressing the VP6. Insect cells were analyzed at 48h post-infection at a MOI of 1. Fluorescence was studied in a confocal microscope.*

The recombinant VHH specific for p54 was efficiently expressed by the baculovirus vector in insect larvae (Figure 8)

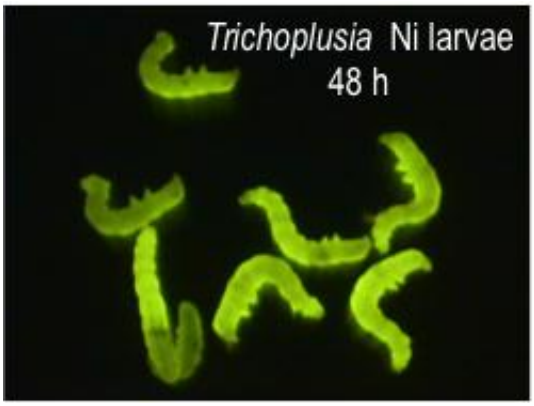



**Figure 8:** *Expression of recombinant fluorescent VHH specific for protein p54 in insect larvae (T. ni).*

Then, we used crude extracts on ASFV-infected Vero cells. Figure 9 shows the specific staining of protein p54 protein in insect cells infected by a recombinant baculovirus expressing p54 and the recognition of this protein induced during infection with ASFV, the last with a similar pattern that shown with a polyclonal antibody from a rabbit immunized with the recombinant p54.

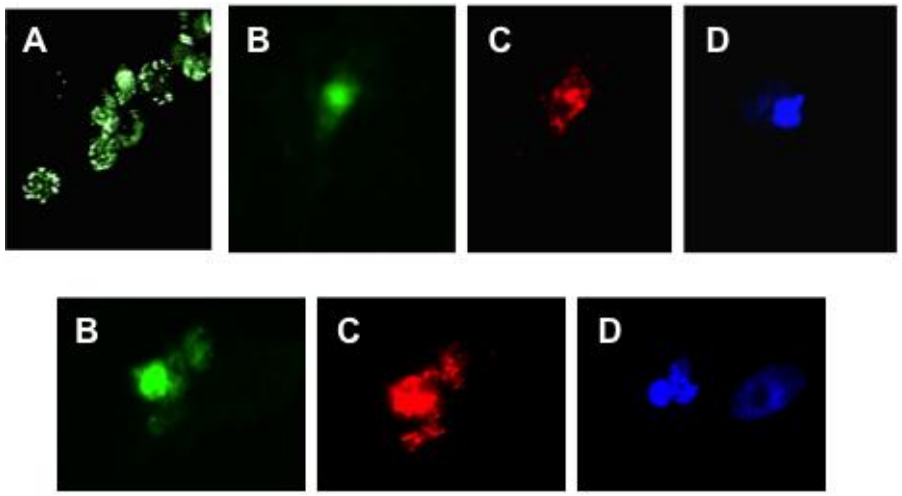



**Figure 9:** *Recombinant fluorescent VHH recognition of protein p54 expressed in insect cells by a recombinant baculovirus* **(A)** *or in ASFV-infected Vero cells* **(B)**. *Fluorescence pattern of p54 in ASFV-infected Vero cells obtained with a rabbit anti-p54 polyclonal serum* ***(C)***. *Hoechst staining of ASFV-infected Vero cells.*

Finally, the insect-derived recombinant fluorescent antibody was used to detect ASFV in tissue samples from a experimentally infected pig. Infected cells were successfully detected in the tissue analyzed (Figure 10).

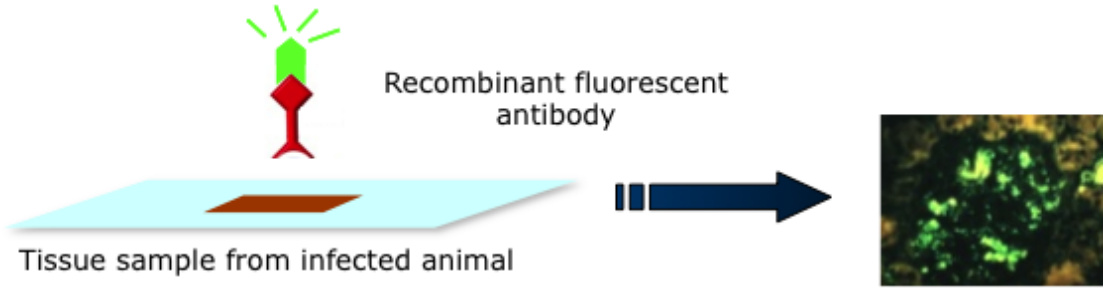



**Figure 10:** *Staining of a kidney section of an ASFV-infected pig with the recombinant fluorescent VHH specific for protein p54.*

**Discussion:**

The present project helped to develop a new generation of reagents for ASFV diagnosis. These reagents consist on recombinant antibodies (single-chain and single-domain) fused to a fluorescent protein (green fluorescent protein). Recombinant antibody clones were obtained from spleen plasma cells of mice immunized with the ASFV protein p54 or from circulating blood plasma cells from immunized llama with the same protein. We have demonstrated that antibodies of this nature (single-chain or single-domain), fused to the green fluorescent protein can be used for detection of specific viral proteins in permeabilized cells. Fluorescent recombinant antibodies were efficiently produced by IBES® technology (insects as living biofactories), a new technology which optimizes the productivity of baculovirus expression system. Furthermore, the VHH fluorescent antibody was able to detect the virus in tissue cuts from a experimentally infectd pig. Those antibodies will be adapted to diagnostic in field pig samples, constituting valuable reagents in the ASFV detection in any diagnostic laboratory and will potentially be considered gold standard tools at any reference laboratory. We will asses the availability of these reagents by their license to a company with the necessary expertise in commercialization to diagnostic laboratories internationally.

The immediate benefit for pork producers in EE.UU. is the availability of the above mentioned reagents in the diagnostic laboratory network in EE.UU. implicated in the diagnostic of exotic diseases and for ASFV in particular. Those reagents are free of any risk of virus transmission because their production does not require the use of infective ASFV as it happened in the past when non-recombinant reagents were used in the diagnostic methods.