RESEARCHREPORT
SWINE HEALTH

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Title: Evaluation of envelope proteins for rapid induction of protective immune response against classical swine fever - **NPB#11-045**

Investigator: Dr. Manuel V. Borca

Institution: Plum Island Animal Disease Center, ARS, USDA

Date Submitted: April 16th, 2013

Industry Summary:

The main objective of this research project was to determine if native and/or modified forms of Classical Swine Fever Virus (CSFV) proteins present in the surface of the virion were able to induce a rapid and protective immune response against CSFV. To accomplish the proposed objective we took two approaches; 1) we assessed the capability each CSFV envelope proteins (E0, E1, and E2) for eliciting a protective immune response against the disease, and 2) we introduced modifications into CSFV envelope proteins to increase their capability of inducing an effective early protection against the virus.

All three proteins (E0, E1, and E2) were produced using a baculovirus/insect cell system to obtain high protein yields. Proteins were purified, tested for purity, combined with adjuvant (Sigma), and used to immunize 30-40 lbs pigs. Animals were inoculated via IM receiving boosters at 28, 42, and 56 days post primo inoculation. All pigs were challenged intra-nasally (IN) 1 week after the last boost. Using this immunization scheme only those animals that were immunized with E0 or E2 elicited high antibody titers and survived the challenge. E1 protein did not elicit a detectable antibody response nor did it elicit a protective immunity in pigs against CSFV. All animals succumbed to challenge.

To further understand the protective efficacy of E0 and E2 proteins we inoculated groups of 4 week-old swine with 1 or 2 doses of each protein. These animals were then exposed to CSFV IN at 21 days after single or last inoculation. All the animals survived the infection, suggesting that lesser doses are sufficient to induce protection. Similarly animals receiving three doses of the proteins and challenged 1 week after last inoculation were protected against CSFV, suggesting that strong response can be induced shortly after 3 inoculations of both proteins.

After the protective efficacy of wild-type E0 and E2 proteins was established, we introduced modifications to these proteins aimed to enhance the immune response against CSFV. E0 and E2 proteins were fused to flagellin; or to single chain anti-class II antibody sequences. Production and purification of modified proteins had to be adjusted from original procedures to obtain high yields of modified proteins. Proteins were then used to assess their capability to induce an antibody response in pigs. Unexpectedly, inoculation of these proteins into swine did not elicit a measurable antibody response suggesting that as formulated E0 and E2 will not induce an efficient immune response in pigs against CSFV. Work will continue to address this problem.

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

For more information contact:

National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

Contact Information:

Dr. Manuel V. Borca
Lead Scientist CSFV and ASFV
Plum Island Animal Disease Center
ARS, USDA
Greenport, NY 11944
USA
Phone: 631 323 3135
E-mail: manuel.borca@ARS.USDA.GOV

Keywords: classical swine fever virus, glycoproteins, vaccines, protection.

Scientific Abstract:

We have analyzed the individual effect of each of CSFV structural proteins (E0, E1, and E2) on the induction of humoral response and protection against the infection. Native version of each of these proteins was produced in baculovirus. Briefly, His-tagged CSFV E0, E1, or E2 genes were synthesized using polymerase chain reaction (PCR) followed by cloning of amplified genes into the pENTR/D-TOPO entry vector (Invitrogen, Carlsbad, CA). Expression of envelope proteins was achieved by cloning those genes into entry vectors suitable for recombination with baculovirus (Invitrogen). Entry vectors were verified for the presence and fidelity of cloned genes by sequencing. Recombinant baculoviruses were generated after recombination of entry clones with linearized baculovirus DNA and transfection of suitable insect cells. Insect cell culture medium containing recombinant viruses were harvested and used to infect fresh insect cells to produce high-titer viral stocks. Infected insect cells were used to analyze the expression of CSFV envelope proteins by Western blot. Expressed envelope proteins were purified using metal ion affinity chromatography on cobalt resin columns. The fractions collected during purification were analyzed by Western blot. Details in the production of recombinant baculovirus, protein expression and their purification are described in Gavrilov *et al*., (2011). Thirty to forty lbs pigs allotted into groups (n=5) were vaccinated intramuscularly with 50 micrograms of purified proteins in water-in-oil emulsions followed by an intranasal challenge with 10^5 TCID50 of highly virulent strain Brescia (BICv) 7 days after the last inoculation. In each experiment, a control group was mock vaccinated and received the challenge as previously described. Blood, nasal swabs, and tonsil scrapings were collected from vaccinated and mock-vaccinated control pigs after challenge. Viral loads in those clinical samples were determined by virus titration in SK6 cells or real time RT-PCR. Serum samples were collected throughout the experiment and the presence of CSFV E0 and E2 antibodies were assessed using commercial ELISA test kits. Neutralizing antibody response was assessed using virus neutralization assays. Detailed information about these experiments could be found in the attached paper (Gavrilov *et al*., 2011). Results demonstrated that as expected, E2 is able to induce a neutralizing antibody response as well as to protect swine against the virulent challenge. Interestingly, purified E0 was also efficient in raising a neutralizing antibody response and protection against the challenge.

In order to increase antigenicity and immunogenicity over wild-type proteins, E0 and E2 were then modified by fusing encoding genes to either an immunostimulatory molecule, flagellin; or to a protein moiety that delivers molecules to professional antigen presenting cells: a single chain anti-class II antibodies. Flagellin is the major structural component of the bacterial flagella. Cells of the innate immune system recognize conserved pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLRs). Flagellin is recognized by TLR5 that signals through TLR5/TLR4, likely activating the interferon pathway. Therefore, flagellin is considered a potent immune activator that rapidly induces expression of proinflammatory cytokines, chemokines, and costimulatory molecules. Flagellin gene was kindly provided by Dr. M.A. Martinez (INIA, Spain) and was synthetically fused to the His-tagged E0 and E2. In addition the efficacy of subunit vaccines might be also improved by using an immunotargeting approach. This system is based on the hypothesis that antigens coupled to monoclonal antibodies specific for class II MHC could be targeted into antigen presenting cells. Here we used a recombinant single chain antibody directed to an invariant epitope of the porcine MHC II DR molecule attempting to target E0 and E2 to cells harboring this invariant MHC Class II epitope. After expression, the antigenicity (i.e.: induction of antibody response) of E0 and E2 was assessed in swine. Pigs, 30-40 lbs (2 animals/group), received 1

dose of E0sc, E0flag, E2sc, or E2flag via IM. Sera were collected from inoculated animals at 7, 14, and 21 days post incoculation and tested by ELISA (Idexx CSFV Ab test). All animals tested negative for CSFV antibodies suggesting that the fusion proteins as designed and synthesized were not antigenic. Further alternatives, including the production of new constructs and changes in the synthesis process, are being pursued at this time.

Introduction: An overview of the researchable question and its importance to producers.

Classical swine fever (CSF), classified as a reportable disease to the OIE (World Organization for Animal Health), is a highly contagious, economically significant viral disease of domestic and wild pigs. The disease, which is often fatal, is characterized by fever, hemorrhages, ataxia and immunosupression; however, the course of infection varies depending on host characteristics and the particular virus strain causing the infection (Van Oirschot, 1999). CSF occurs in several forms, ranging from highly lethal to subclinical. (Van Oirscht, 1999; Wengler et al., 1995). The causative agent, Classical Swine Fever Virus (CSFV), is a member of the genus *Pestivirus* of the family *Flaviviridae* (Wengler et al., 1995) along with two other viruses of significant veterinary importance, bovine viral diarrhea virus (BVDV) and border disease virus (BDV).

CSF is enzootic in Eastern Europe, Southeast Asia, Central and parts of South America, southern Mexico, and the Caribbean. The United States was declared free of CSF in 1978. Recent outbreaks in countries free of the disease, including the Netherlands, Germany, England and Spain, have resulted in highly significant economic losses for their respective swine industries (Meuwissen et al. 1999). A disease control policy involving slaughter of exposed animals has been practiced in countries of the European Union. **Controlling and eliminating the disease by this approach has proven effective, but has resulted in staggering economic losses for both governments and swine producers alike. Costs related to the recent outbreak in the Netherlands (1997-1998) exceeded two billion dollars (Pluimers et al. 1999).**

Vaccination has demonstrated to be one of the most efficacious tools for eradication and control of CSF. Live attenuated vaccines (LAVs) against CSF of proven efficacy and safety have been used worldwide and administered parenterally to domestic swine or orally to wild pigs always demonstrating superior effectiveness (van Oirschot 2003). Due to the rapid onset of immunity induced by LAVs against CSFV, vaccination might be a key option to control spreading of the disease should it occur in a country free of the disease, i.e.: United States. A major disadvantage of implementing emergency vaccination with these vaccines is that they lack DIVA (differentiation of infected from vaccinated animals) capabilities. This feature poses a significant drawback since testing for CSF status is based on the detection of antibodies against CSFV E2 protein. Hence, vaccinated and exposed animals will test equally positive in this test. This is one of the reasons leading to a non-vaccination/culling strategy policy applied by European Union countries in the past. Exposed herds were eliminated regardless the status of the infection. To avoid the potentially high economic burden of those control measures, a vaccination-to-live strategy using DIVA vaccines followed by elimination of infected animals could minimize significantly those losses. Today, such DIVA vaccines for CSF are not available in the market.

In order to overcome the inherited problems of currently available LAVs, subunit vaccines with DIVA capabilities are another interesting tool that can be used to control CSF. Subunit vaccines based on baculovirus-expressed CSFV E2 protein and the companion serologic tests for detection of antibodies against naturally infecting CSFVs have been developed and have been marketed in several countries around the world. Two major problems arise with these vaccines, 1) a limited protective efficacy particularly before 14 days post vaccination (Moormann et al., 2000; Uttenthal et al., 2001; Risatti et al.,2005b); and 2) the poor discriminatory capability of the DIVA companion test (Floegel-Niesmann, 2001). These serologic tests detect antibodies against CSFV E^{rns} protein to denote the presence of natural infection in affected herds. E^{rns} antibody detection tests have poor sensitivity leading to a high rate of false negative results and misdiagnosis of CSF.

The lack of induction of a rapid onset of immunity in vaccinated pigs is the major limitation of these vaccines. None of the commercial subunits vaccines were able to protect pigs at 7 days post vaccination when pigs were challenged with Haiti 96 a moderately virulent CSFV that circulated in the Caribbean region in the 90's (Risatti et al., 2005b). Recent studies have aimed to solve the limited protective efficacy of CSFV E2 subunit vaccines. Pigs vaccinated with a water-in-oil emulsion of E2 glycoprotein produced in the mammary gland of adenoviral transduced goats (Toledo et al., 2008), survived a challenge at 7 days post-vaccination when exposed to a virulent CSFV strain (Barrera et al., 2010). Although in that study, pigs showed signs of CSF characterized by mild fever, depression, anorexia, and a short period of viremia.

At necropsy some pigs showed hemorrhagic lymph nodes and spleens. In a follow-up study (Toledo et al., 2010), demonstrated that a human α-interferon (hINFα) increases the immunogenicity of E2 glycoprotein produced in goat milk. Pigs vaccinated with this co-formulation of E2-hINFα were protected against clinical signs of the disease and viremia as early as 7 days post-vaccination. Although these are proof-of-concept studies, they clearly show that the efficacy of subunits vaccines can be improved.

Objectives:

Although currently available live attenuated vaccines are efficacious; safe and rationale designed subunit vaccines with DIVA capabilities (marker vaccine), that elicit a rapid onset of immunity (within a week of vaccination) will contribute to better management of a disease outbreak in United States should it occur.

Due to its immunogenic features, subunit vaccines against CSFV have been developed exclusively based on E2 glycoprotein. However, the immunogenic capabilities of E0 and E1 glycoproteins expressed as subunits, and their overall contribution to immunity against CSFV remain unknown. Thus combinations of E2 with the other envelope proteins may elicit a better protective response, in which case a differential serologic test can be formulated by using other CSFV proteins as targets i.e.: NS3.

Immunostimulating cytokines eliciting enhanced immune responses when combined with subunit vaccine candidates have shown interesting results. A simplified approach that includes expression of CSFV envelope proteins as fusion with immune stimulators may elicit a rapid immune response against the virus in vaccinated pigs.

Thus, the objective of this research project was to evaluate native and modified forms of CSF envelope proteins for their capacity to induce rapid protective immune response against the CSFV.

Materials & Methods

Cells and viruses
Propagation of recombinant baculoviruses and expression of the envelope proteins were performed in *Spodoptera frugiperda* derived Sf9 cell line. Sf9 cells were maintained in adherent cultures in Grace's insect cell culture medium (TNM-FH) supplemented with 10% fetal bovine serum and gentamicin (Lonza, Walkersville, MD).
SK6 cells (Terpstra et al., 1990), free of BVDV, were used to propagate BICv (Risatti et al., 2005). SK6 cells were cultured in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Lonza).
For titration of CSFV from clinical samples, SK6 cells in 96-well plates (Corning, Lowell, MA) were infected and incubated at 37 °C and 5 % CO_2 for 4 days. Viral infectivity was detected using an immunoperoxidase assay with the anti-E2 CSFV monoclonal antibody (mAb) WH303 (Edwards et al., 1991) and Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The plates were read microscopically for stained cells. Titers were calculated according to the method of Reed and Muench (Reed and Muench, 1938) and were expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml. As performed, test sensitivity was $\geq \log_{10}$ 1.8 $TCID_{50}$/ml.

2. **Generation of entry clones and recombinant baculoviruses**

Polymerase chain reaction (PCR) was utilized to synthesize the E0, E1, and E2 genes using appropriate primers. Each reverse primer included six histidine residues coding for a tag at the C-terminus of the respective protein to allow purification by immobilized-metal affinity chromatography. Forward primers of the genes encoding the three envelope proteins included additional sequence which coded for their putative signal peptide (Rumenapf et al., 1993; Ruggli et al., 1995; Moser et al., 1996; van Rijn et al., 1996) or human CD33 signal peptide, whereas forward primers used to amplify non-glycosylated forms of the envelope proteins did not include signal peptide sequences. The E2 gene was synthesized without its transmembrane region (Hulst et al., 1993) to facilitate its subsequent expression and purification. The PCR conditions were as follows: incubation at 95°C for 2 minutes, 35 cycles of 95°C for 30 s, 58°C for 30 s, and 68°C for 2 minutes, and final extension step of 68 °C for 3 minutes. The amplification was undertaken in a thermocycler (Mastercycler, Eppendorf, Westbury, NY). The resulting PCR fragments were eluted from a 1% agarose gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA), and cloned directionally into pENTR/D-TOPO entry vector (Invitrogen). To confirm that inserted genes were in frame, entry clones were verified by automatic sequencing.

Similarly, genes encoding E0 and E2 proteins were fused in frame to flagellin (flag) or MHC-II single chain antibody (sc) encoding ORFs to obtain modified E0flag, E0sc, E2flag, and E2sc.

Recombinant baculoviruses were produced using the BaculoDirect C-Term expression system (Invitrogen) according to the manufacturer's instructions. Briefly, recombinant baculoviruses expressing envelope proteins were constructed by attL-attR reaction between entry clones and BaculoDirect C-term Linear DNA (derived from *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV)) containing Herpes Simplex Virus thymidine kinase gene located between recombination sites for negative selection with ganciclovir. Transfection and subsequent propagation of recombinant baculoviruses was performed in Sf9 cells as recommended by the manufacturer using Cellfectin (Invitrogen). The cells were grown until signs of infection were observed (5 – 7 days) and the resulting stock (P1) was harvested and amplified once by infecting confluent Sf9 cells to obtain a high-titer viral stock (P2, $\geq 5 \times 10^7$ pfu/ml).

3. Detection and purification of recombinant proteins

To determine the optimal time for expression of the recombinant proteins, confluent Sf9 cells (about 1.5×10^6/ml) in 75 cm^2 flasks were infected with high titer recombinant baculovirus suspension (P2 stock) at multiplicity of infection (MOI) of 5 to 10. Insect cells and culture medium were harvested at various time points after infection. Medium was centrifuged at 2000 × g for 5 min at 4 °C and cell pellet was washed twice with phosphate-buffered saline (PBS) followed by resuspension in RIPA buffer (Sigma Aldrich, St. Louis, MO). After incubation for 10 min on ice, the preparations were clarified by centrifugation at 16,100 x g for 10 min. Expression of the recombinant envelope proteins was verified by western blot analysis. Briefly, proteins from culture medium and cell lysates were separated under reducing conditions on a 10% NuPage Novex Bis-Tris gel (Invitrogen) by a discontinuous SDS-PAGE system and transferred to polyvinylidene fluoride (PVDF) membrane (Invitrogen). MagicMark XP Protein Standard (Invitrogen) was used as a molecular weight standard. The membrane was blocked and incubated with anti-his C-term antibody (Invitrogen) at a dilution of 1:5000 for overnight. The membrane was washed and incubated with goat anti-mouse IgG antibody conjugated with alkaline phosphatase (WesternBreeze Chemiluminescent Detection Kit, Invitrogen). Finally, the membrane was developed using CDP-star chemiluminescent substrate provided with the kit and subsequently exposed to an X-ray film (Kodak X-OMAT LS film, Kodak, Rochester, NY) (Figures 1 and 2).
The cell lysates were applied to HisPur Cobalt Resin Columns (Thermo Fisher Scientific, Rockford, IL) and purified following the manufacturer's instructions. The collected fractions were analyzed by western blotting as described above and Coomassie blue staining. For Coomassie blue staining, after separation of proteins on 10% Bis-Tris gel, the gel was stained with Coomassie blue stain (Simply Blue SafeStain, Invitrogen).

Protein concentration was determined by (measured with) BCA Protein Assay (Thermo Fisher Scientific) against bovine serum albumin as standard at OD of 595 nm using NanoDrop (NanoDrop Technologies, Wilmington, DE).

4. Animal experiments: screening of wild type proteins

For initial screening, twelve 30-40 lbs commercial breed pigs were allocated randomly into groups of two animals each, and pigs were inoculated with E0, E1, or E2 proteins. Purified envelope proteins were mixed with Sigma Adjuvant System (Sigma-Aldrich, St. Louis, MO) according to instructions given by the manufacturer to obtain a water-in-oil emulsion. One milliliter of each vaccine containing ~80-120 µg of purified protein was injected via intramuscular route into the neck of animals. Pigs were boosted with the same dose 4, 6, and 8 weeks later. Blood samples were collected from each animal weekly starting from the day of first inoculation. CSFV antibodies (E0 and E2 antibodies) were detected using commercially available ELISA tests (Idexx). Antibodies against E1 were tested by seroneutralization assays (see above).

A similar screening was performed with modified E0 and E2 proteins named E0sc, E0 flag, E2sc, E2flag. Eight 30-40 lbs commercial breed pigs were allocated randomly into groups of two animals each. In this case animals received 1 dose of one milliliter of each protein formulation containing ~80-120 µg of purified protein that was injected via intramuscular route into the neck of animals. Sera was collected at 7, 14 , and 21 days post inoculation. The presence of CSFV antibodies were detected using commercially available ELISA tests (Idexx).

After antibody detection, challenge infection with 10^5 50% tissue culture infective dose ($TCID_{50}$) highly virulent BICv was done intranasally 1 week after the last inoculation. After challenge, pigs were observed daily for three weeks for signs of CSF – such as, anorexia, depression, fever – defined as body temperature above 104.0 °F, purple skin discoloration or

hemorrhagic diathesis, staggering gait, diarrhea, and cough. Body temperatures were recorded daily throughout the experiment.

5. Enzyme-linked immunosorbent assay (ELISA) for detection of anti-E^{rns} and anti-E2 antibodies

Serum samples were studied for the presence of CSFV E^{rns} specific antibodies with IDEXX CHEKIT-CSF-MARKER ELISA Test Kit (Idexx Laboratories, Schiphol-Rijk, the Netherlands), and for detection of CSFV E2 antibodies with IDEXX CSF SERO Antibody ELISA Test Kit (Idexx Laboratories). ELISA test kits were used according to the protocols provided by the manufacturer.

6. Seroneutralization assays

Sera samples were heat inactivated at 56 °C for 30 min prior to performing the serum neutralization assay. Two-fold serial dilutions were prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and mixed with equal volume of BICv or glycosylation mutant viruses (Risatti et al., 2007; Fernandez-Sainz et al., 2008) at final concentration of 10^2 virus particles per well. The mixtures were incubated for 1 hr at 37 °C and 5 % CO_2 and then transferred to 96-well flat-bottom tissue culture plates (Corning) followed by addition of SK6 cells (1 x 10^4 per well) which has been seeded 48 hr earlier. The plates were incubated at 37 °C and 5 % CO_2 for 4 days. The supernatant was removed from each well and the cells were fixed with methanol-acetone (50 % vol./vol.) solution and air-dried. Plates were stained by immunoperoxidase assay. Briefly, plates were blocked with blocking solution (complete DMEM and 2 % BSA) for 30 min at 37 °C and incubated with primary mAb WH303 at a dilution of 1:500 for 30 min at 37 °C. 50 µl of goat anti-mouse IgG secondary antibody at 1:400 dilution was added to each well and the plates were incubated for 30 min at 37 °C, and washed twice with PBS. The plates were stained with peroxidase (Vecstatin ABC Kit, Vector Laboratories, Burlingame, CA). Neutralizing antibody titers were expressed as the reciprocal of the highest two-fold serum dilution neutralizing approximately 100 $TCID_{50}$ of CSFV in 50% of the wells (Reed and Muench, 1938).

Results:

To investigate the immunogenicity and protective efficacy of the three CSFV envelope proteins, pigs were vaccinated intramuscularly with oil-in-water emulsion containing purified forms of each CSFV envelope protein. Animals were boosted at day 28, 42, and 56 after the first inoculation (Table 1). No local or systemic reactions as well as elevated body temperature were observed after inoculation with the protein preps. Mean temperatures before and after inoculation were in the range 101.8-104.0 °F.

Antibody response to vaccination: Anti-E0 or E2 antibodies were first observed by day 21 post-inoculation with higher titers at 1 week after the second vaccination (Figure 3). Non-vaccinated control pigs were negative for E0 or E2-specific antibodies. **E0 and E2 induced a long-lasting neutralizing antibody response.** Serum neutralizing antibody (NtAb) titers were observed weekly following the first inoculation. First detectable NtAb levels against CSFV strain Brescia were observed at 7 days post-second vaccination. While in the group vaccinated with E0, neutralizing antibodies against CSFV strain Brescia were detected starting at 14 days after the second inoculation. No detectable antibodies against CSFV Brescia were observed in the group incoculated with E1 glycoproteins either by ELISA or seroneutralization.

To assess the ability of CSFV envelope proteins to induce protection against infection with virulent BICv, pigs were challenged intranasally with 10^5 $TCID_{50}$ of CSFV strain Brescia 1 week after the last inoculation (Table 3). Only those animals that were vaccinated with E0 or E2 proteins developed protection and survived infection with CSFV, without showing clinical symptoms. Instead animals vaccinated with E1 glycoprotein were not protected and developed anorexia, depression, and fever by 3-4 days post-challenge (dpc) and died within 6-14 dpc (Fig. 2.9A and B, and Table 2.3) suggesting that elicitation of detectable antibody titers is required for protection against the virus. As expected, non-vaccinated control pigs receiving BICv only (n=2) also showed clinical signs of CSF and died within 10 dpc (Fig. 2.9B and Table 2.3).
Viremia and virus shedding were measured at 0, 3, 7, 10, 14, 21, and 28 dpc. While all groups of animals were viremic at 3 dpc, high levels of viremia were observed at 7 dpc in non-vaccinated controls and in animals inoculated with E1 protein, with virus titers remaining high until their death. Virus was also detected in nasal swabs and tonsil scrapings of non-vaccinated controls at 7 and 10 dpc indicating that these animals were shedding large amounts of CSFV. Low levels of viremia were observed in E0 or E2-vaccinated pigs at 7 dpc that tend to disappear towards 10 dpc.

To investigate the immunogenicity and protective efficacy of the three modified CSFV envelope proteins (E0sc, E0flag, E2sc, and E2flag), pigs(n=2) received 1 dose intramuscularly of a oil-in-water emulsion containing purified forms of each modified protein (Table 2). No local or systemic reactions as well as elevated body temperature were observed after inoculation with the protein preps. Mean temperatures before and after inoculation were in the range 101.8-104.0 °F. Serum was obtained from these pigs at 0, 7, 14, and 21 days post vaccination and assessed for CSFV antibodies by ELISA.

Antibody response to vaccination: Anti-E0 or E2 antibodies were not detected at any time point after vaccination with modified protein (Figure 4, E2 ELISA shown, E0 data not shown). Similarly non-vaccinated control pigs were also negative for E0 or E2-specific antibodies. As observed before with animals inoculated with E1 glycoprotein lack of antigenicity correlated with lack of protection against CSFV. The inability of the modified E0 and E2 proteins to induce a detectable antibody response might be linked to aspects related with synthesis and purification of proteins. These aspects are being carefully reassessed before arriving to a final conclusion on the utility of these modifications while trying to achieve protection against CSFV.

Figure 1. Western blot analysis of baculovirus expressed CSFV envelope proteins E0, E1, and E2. CSFV proteins were detected using an anti-His monoclonal antibody. All proteins were 6X –histidine tagged for purpose of purification when using affinity chromatography (Nickel columns). P: PNGaseF glycosidase treatment was used to determine that baculovirus expressed proteins were glycosydated.

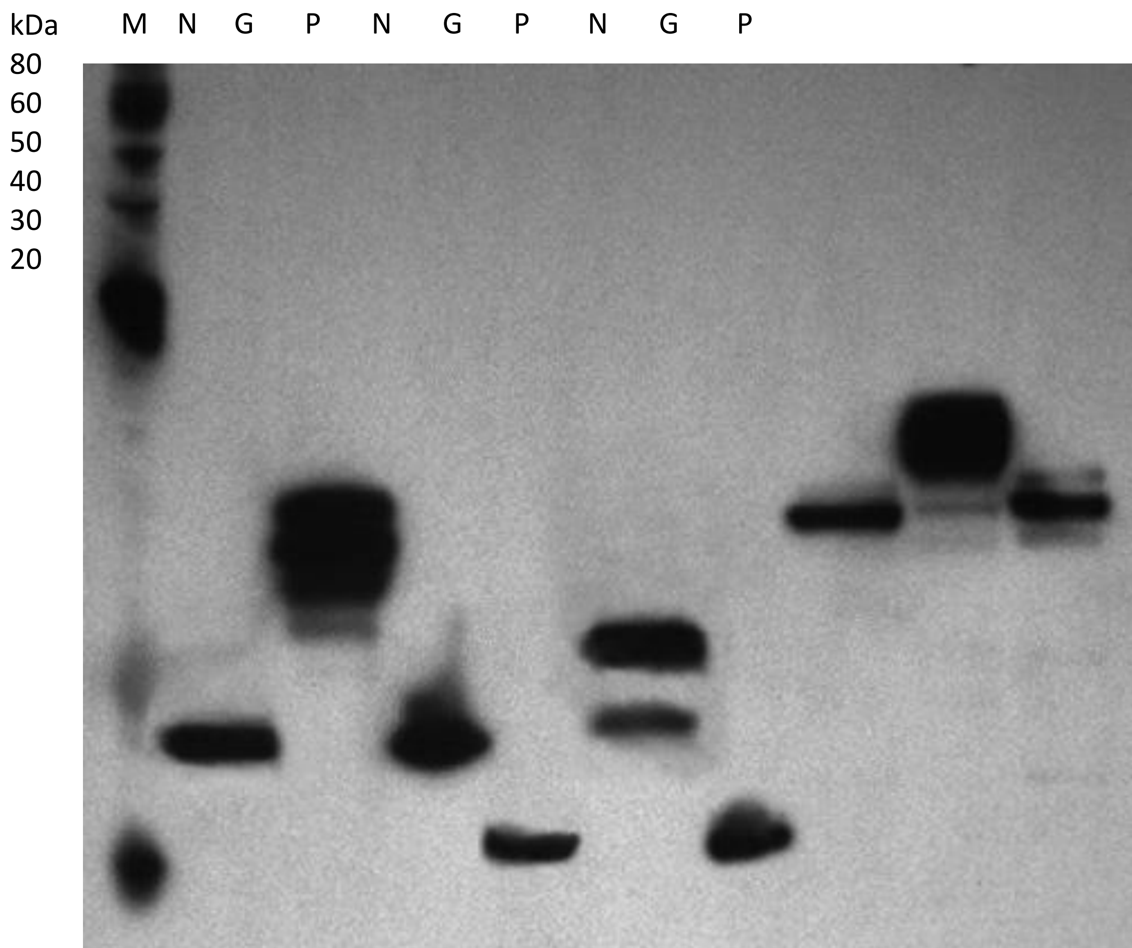


Figure 2. Expression of modified CSFV E0 and E2 glycoproteins using a baculovirus-insect cells system. (A) coomasie blue staining, (B) western immunoblot.

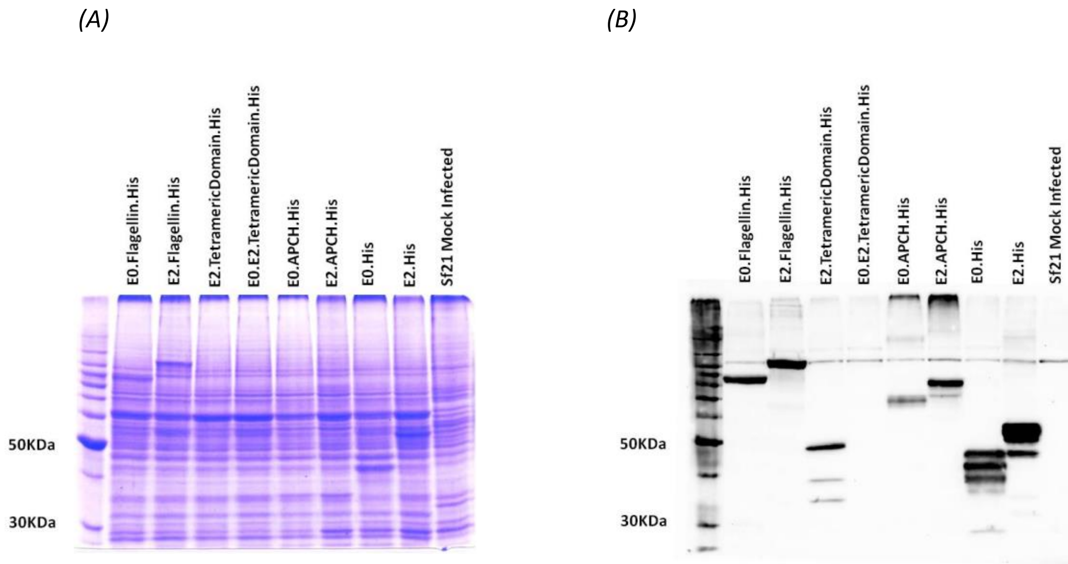


Table 1: Vaccination schedule for animals inoculated with CSFV E0 and E2 proteins. These animals were challenged with CSFV strain Brescia (highly virulent). All animals were inoculated via IM with proteins in an oil-in-water emulsion (Sigma Adjuvant, Sigma).

Days	0	7	14	21	28	35	42	49	56	63	70	77	84
Vaccination													
Challenge													
Temperature													
Sera samples													
Clinical observation													

Table 2: Vaccination schedule for animals inoculated with modified CSFV E0 and E2 proteins (E0sc, E0flag, E2sc, E2flag). These animals were challenged with CSFV strain Brescia (highly virulent). All animals were inoculated via IM with proteins in an oil-in-water emulsion (Sigma Adjuvant, Sigma).

Days	0	7	14	21
Vaccination				
Temperature				
Sera samples				
Clinical observation				

Figure 3. Development of antibodies against CSFV in pigs inoculated with E0 (A), E2 (B), and E1 (C) proteins (A and B respectively). Note: two independent studies shown. E1 inoculated pigs did not elicited a neutralizing antibody response against CSFV.

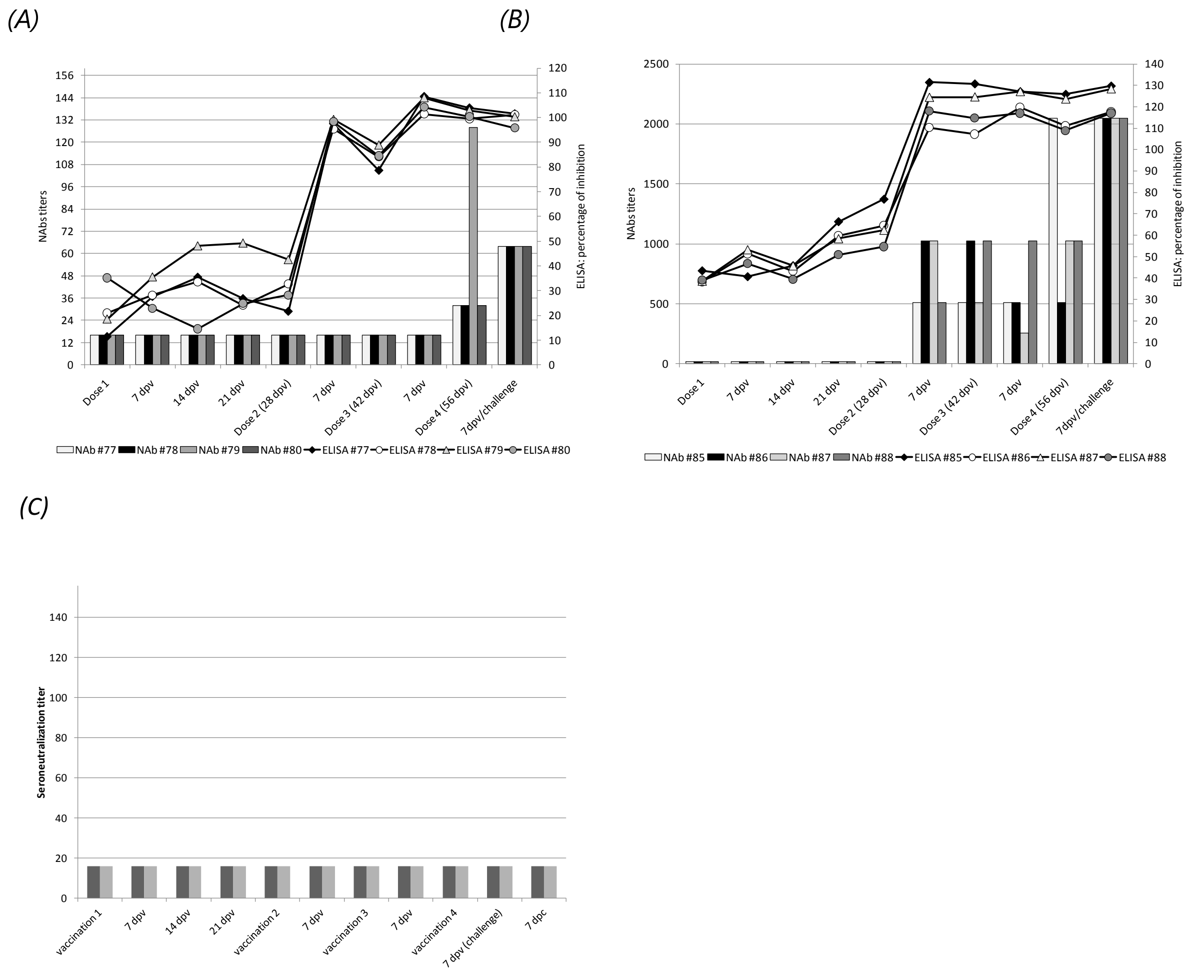
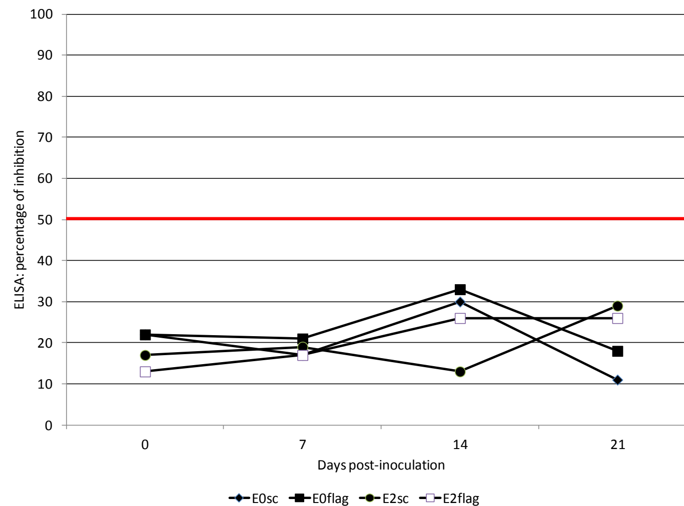


Table 3. Protection induced in inoculated pigs by CSFV E0, E1, and E2 glycoproteins.

Pig number	Survivors/total	Time of death (dpc)	Time of the onset of fever (dpc)	Duration of fever (days)	Maximun Temperature (F)
E0	4/4	0	0	0	104.4
E1	0/2	8	2	6	107
E2	4/4	0	6	3	105.8
Control	0/2	8	4	4	107

Table 4. Development of antibodies (CSFV E2 ELISA) against CSFV in pigs inoculated with modified CSFV envelope proteins (E0sc, E0flag, E2sc, E2flag). Cut-off value= 50%.



Discussion:

In this study we wanted to assess the ability of modified CSFV envelope proteins E0 and E2 to induce humoral immune responses, and the ability to induce protection in swine against challenge with highly virulent CSFV.
While E0 and E2 proteins, but not E1, were able to induce a sustained humoral response against CSFV and induce protection against the virus; the modified counterparts, as expressed here, failed to induce a measurable antibody response.

Although this approach has been proven effective to increase immunogenicity with other antigens, we concluded that the lack of antigenicity of modified E0 and E2 proteins fused to immunostimulatory proteins observed here, might be linked to aspects related with synthesis and purification of proteins. These aspects are being carefully reassessed before arriving to a final conclusion on the utility of these modifications while trying to achieve protection against CSFV.

In summary, effective subunit vaccines, with inherited DIVA capabilities (differentiation of infected from vaccinated animals), that are able to elicit rapid protection against CSFV are highly desirable. Vaccines like this will be an added tool for controlling CSFV should an outbreak occur in the US.