RESEARCH REPORT
SWINE HEALTH

Title: Identification of host factors interacting with classical swine fever virus proteins: development of novel anti-viral therapeutics." **NPB# 09-111**

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Industry Summary: During the infection of a cell, a virus gets in contact with many host proteins. These interactions between virus and host factors enable the virus the successful production of progeny and progress of the disease. Identification and characterization of such interactions could be useful in providing novel alternatives to alter virus multiplication and, perhaps, disease. This project proposed the identification of swine proteins interacting with classical swine fever virus (CSFV) proteins during the infection. Results obtained enable the identification of several host proteins interacting with CSFV structural protein Core. Core protein is the major contributor to the virus capsid. Several of these interactions have been studied in detail and the regions of the CSFV Core protein interacting with the host proteins were identified. Mutant CSFV viruses having altered these regions have been demonstrated that have severely altered their ability to produce disease in swine. Therefore, the manipulation of the identified host-virus interactions allowed the development of attenuated strains of virus which may constitute a tool for the further development of live attenuated vaccine against classical swine fever. Additionally, this knowledge may open the possibility of designing bio therapeutic compounds that could alter those critical interactions that may limit the spread of the disease.

Key Words: Classical Swine Fever, virus proteins, anti-viral therapeutics, novel vaccines, host factors, Pathogenesis.

Scientific Abstract: Classical swine fever (CSF), classified as a notifiable disease to the OIE (World Organization for Animal Health), is a highly contagious, economically significant viral disease of domestic and wild pigs. The causative agent, classical swine fever virus (CSFV), is a member of the genus *Pestivirus* of the family *Flaviviridae*. Viral mechanisms involved in CSFV induction of disease, generalization of infection, tissue tropism, host range, and induction of immune responses are not well understood. The spread of CSFV into non-enzootic regions with high-density pig farming is of major concern. Current vaccines, either lack antigenic markers, a fact that impedes their use during a disease outbreak in CSFV free areas, or are poor inducers of early immunity, a critical factor during a disease outbreak. Therefore, it is important to explore the possibility of developing novel approaches to prevent or restrain the progress of the infection in the affected animal. The main goal of this project is to identify host proteins directly involved in the process of virus infection. Currently, there is almost no information available regarding host factors directly interacting with the CSF viral proteins during its replication cycle. This study should contribute in the understanding of the pathogenesis of the disease and provide valuable

information for the development of novel alternatives to control (such as novel vaccines and anti-viral therapeutics) the spread of the infection in the natural host. We specifically focus in the identification of host proteins interacting with virus structural proteins (SP) using as a tool a yeast two-hybrid system (YTH). The YTH as used for screening a swine primary macrophage cDNA library to identify CSFV SP-host protein-protein interactions. Structural components of the CSFV virion include the nucleocapsid Core protein and envelope glycoproteins E^{rns}, E1, and E2. We have screened all 4 virus SP. We have identified 11 host proteins interacting with CSFV Core protein and over 50 others interacting with E2. Technical limitations of the YTH system made difficult to obtain clear results with E^{rns} and E1. Three of the 11 Core binding host proteins were selected for further work: Two of these proteins are involved with the cellular sumoylation pathway, named SUMO-1 (small ubiquitin-like modifier) and UBC9, a SUMO-1 conjugating enzyme. The third host protein is named IQGAP. In all three cases critical amino acid residues in Core protein interacting with each of the host proteins were identified using sets of Core proteins harboring punctual amino acid substitutions in designed areas. Once the critical binding areas in Core protein were identified, to further assess the role of these three host protein interactions in the CSFV growth cycle, alanine substitutions in those critical areas were introduced in Core gene of highly virulent CSFV strain Brescia (BICv). Mutant virus harboring mutation that affect interactions between Core and the host proteins were attenuated in swine. Interestingly, data demonstrated a clear correlation between the failure of Core protein on binding SUMO-1, UBC9 or IQGAP and the attenuation of the virus in swine. These results strongly suggest that interaction of CSFV Core protein with host SUMO-1, UBC9 or IQGAP are a critical step in the pathogenesis of CSF in swine.

Introduction: Classical swine fever (CSF) is a highly contagious, often lethal disease of domestic and wild swine. CSF remains a significant threat to swine industries worldwide, especially to those present in disease free areas. Therefore, it is important to explore the possibility of developing novel approaches to prevent or restrain the progress of the infection in the affected animal. The main goal of this project is to identify host proteins directly involved in the process of virus infection. Currently, there is almost no information available regarding host factors directly interacting with the CSF viral proteins during its replication cycle. This study should contribute in the understanding of the pathogenesis of the disease and provide valuable information for the development of novel alternatives to control the spread of the infection in the natural host. Additionally, the information obtained here may well have a direct application for other Pestivirus with importance in animal health as bovine viral diarrhea virus.

Objectives: These are the specific objectives of the project: (i) Utilizing a two hybrid screening system, individual CSFV structural proteins will be used as bait to identify swine cell proteins that physically interact with them. (ii) The identified host and viral factors will be analyzed in their binding characteristics. (iii) Mutant viruses, having altered their putative binding motif will be produced and characterized *in vitro* and *in vivo*, especially in their ability to cause disease in swine, compared with their parental virulent virus.

Materials & Methods:

Viruses and cells

Swine kidney cells (SK6), free of BVDV, were cultured in Dulbecco's Minimal Essential Media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO). CSFV strain Brescia was propagated in SK6 cells and used for the construction of an infectious cDNA clone (IC). Growth kinetics was assessed on primary swine macrophage cell cultures prepared as described. Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). Viral infectivity was detected, after 4 days in culture, by an immunoperoxidase assay using the CSFV monoclonal antibody WH303 and the Vectastain ABC kit (Vector Laboratories,

Burlingame, CA). Titers were calculated using the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was $\geq$ 1.8 $TCID_{50}$/ml.

Construction of CSFV mutants

A full-length IC of the virulent CSFV Brescia strain (pBIC) was used as a template in which putative SUMO, UBC9 and IQGAP binding sites, in the Core protein were mutated. In all cases native amino acid residues were substituted by alanines (Ala or A). SUMO binding sites were predicted using the SUMOplot Analysis Program (www.abgent.com/tools/SUMOplot) with the consensus sequence ΨKxD/E/P/G (where Ψ is a bulky hydrophobic residue) within CSFV strain Brescia Core protein (Fig. 3 and 4). IQGAP1 binding sites were predicted using the Clustal W Analysis Program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) taking as template IQGAP1 binding areas described for Moloney murine leukemia virus (MMLV) matrix (M) protein (Fig. 3). All amino acid substitutions were introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) performed per manufacturer's instructions and using the following primers (only forward primer sequences are shown): CoreΔS179: 5'-ggtgcaagtgcaagtaaagaggcgaaaccagataggatc aacaa-3'; CoreΔS180: 5'-gcaagtgcaagtaaagagaaggcaccagataggatcaacaaggg-3'; CoreΔS220: 5'-ggaagggtaaaat accaggtcaaagcgaaaggtaaagttaagggaaagaat-3'; CoreΔS221: 5'-aggagtaaaataccaggtcaaaaaggcaggtaaagttaagg gaaagaatacc-3'; CoreΔS246: 5'-aataaaccaccagaatctagaaggcattagaaaaagccctattggcatg-3'; CoreΔS179/180: 5'-ttgtagtggaaggagtaaaataccaggtcaaagcggcaggtaaagttaagggaaagaatacc-3' CoreΔS220/221: 5'-gatggtgcaagtgca agtaaagaggggcaccagataggatcaacaagggtaa-3'; and CoreΔS179/180/220/221: 5'-ttgtagtggaaggagtaaaatacca ggtcaaagcggcaggtaaagttaagggaaagaatacc-3' and 5'-gatggtgcaagtgcaagtaaagaggcggcaccagataggatcaacaagggt aa-3'.

***In Vitro* Rescue of CSFV Brescia and mutant viruses**

Full-length genomic clones were linearized with *SrfI* and *in vitro* transcribed using the T7 MEGAscript system (Ambion, Austin, TX). RNA was precipitated with LiCl and transfected into SK6 cells by electroporation at 500 volts, 720 ohms, 100 watts with a BTX 630 electroporator (BTX, San Diego, CA). Cells were seeded in 12-well plates and incubated for 4 days at 37°C and 5% CO_2. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at -70°C.

DNA Sequencing and Analysis

Full-length clones and *in vitro* rescued viruses were completely sequenced with CSFV-specific primers by the dideoxynucleotide chain-termination method. Viruses recovered from infected animals were sequenced in the mutated region. Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on an ABI PRISM 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3. The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Development of the cDNA library

A porcine macrophage cDNA expression library was constructed (Clontech, Mountain View, CA) using monocytes/macrophages obtained from healthy non-infected forty-pound swine. Macrophage cultures were prepared from defibrinated swine blood. Cells were cultured in plastic Primaria tissue culture flasks (BD Falcon, Franklin Lakes, NJ) in Roswell Park Memorial Institute-1640 medium (RPMI 1640,

Invitrogen, Carlsbad, CA) containing 30% v/v L929 cell supernatant, 20% fetal bovine serum (Invitrogen, Carlsbad, CA), and antibiotics-antimycotics (Invitrogen), for 48 hours (37°C in 5% CO_2). Adherent cells were detached from the plastic by using 10 mM EDTA in phosphate-buffered saline and then reseeded into Primaria 6-well dishes at a density of 5 x 10^6 cells per well and incubated for an additional 24 hours at 37°C in 5% CO_2. Cells were detached using 10 mM EDTA in phosphate-buffered saline, spun at 500x*g* for 10 minutes, with subsequent total RNA extraction using an RNeasy Mini kit (Qiagen, Valencia, CA). Contaminant genomic DNA was removed by DNase treatment using TURBO DNA-*free* (Ambion, Austin, TX). After DNase treatment, genomic DNA contamination of RNA stocks was assessed by real-time PCR amplification targeting the porcine β-actin gene. RNA quality was assessed by using RNA nano chips on an Agilent Bioanalyzer 2100 (Germany). Cellular proteins were expressed as GAL4-AD fusion proteins while CSFV proteins were expressed as GAL4-BD fusion proteins.

Library screening

The GAL4-based yeast two-hybrid system provides a transcriptional assay for detection of protein-protein interactions. The 'bait' protein, CSFV strain Brescia Core protein, was expressed with an N-terminus fusion to the GAL4 Binding Domain (BD). Full-length Core protein (amino acid residues 168-268 of the CSFV polyprotein) was used for screening and for full-length mutant protein construction. The Core fragments used for mapping were made using amino acids 168-212 for the amino half and amino acids 211-268 for the carboxyl half. As 'prey', the previously described swine macrophage cDNA library containing proteins fused to the GAL4-AD was used. Histidine and adenine reporter genes were used for growth selection in addition to two color selection genes, X-gal ($blue^+$/$white^-$) and X-α-gal ($blue^+$/$white^-$). The swine macrophage library used here contains 3 x 10^6 independent cDNA clones. To screen, yeast strain AH109 (Clontech, Mountain View, CA) carrying Core protein was transformed with library plasmid DNA with subsequent selection on plates lacking tryptophan, leucine, histidine and adenine. Tryptophan and leucine are used for plasmid selection, while histidine and adenine are used for identification of positive interacting library fusions. Once identified the positive library plasmids were recovered in *E. coli* and sequenced to identify the cellular interacting protein. Sequence analysis also determined if the library proteins (cellular) were in-frame with the activation domain. To eliminate false positive interactions all library-activation domain fusion proteins were retransformed into strains carrying the viral protein-binding domain fusion proteins, as well as into strains carrying a lam-binding domain fusion, with vectors that only contain the binding domain as negative controls. Lam is Human Lamin C, commonly used as a negative control in the yeast two-hybrid system as Lamin C does not form complexes or interact with most other proteins; however, some studies have shown Lamin C to interact with UBC9, thus binding domain-only vector was used for this purpose. The SUMO-1 recovered from the library contained amino acids (29-101) of porcine SUMO-1 (NCBI Reference Sequence: NP_001106146.1) amino terminal fused to the GAL4 activation domain. The UBC9 protein recovered was similar to *homo sapiens* UBC9 (93% identity) and contained amino acids 2-172 of *homo sapiens* UBC9 (BAD92225) amino terminal fused to the GAL4 activation domain. The IQGAP1 recovered from the library contained amino acids (502-704) of homo sapiens IQGAP1 (NCBI Reference Sequence: AAI39732) amino terminal fused to the GAL4 activation domain

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Animal infections

Each of the mutant viruses was initially screened for its virulence phenotype in swine relative to the virulent strain Brescia. Swine used in all animal studies were 10 to 12 weeks old, forty-pound commercial breed pigs inoculated intranasally with 10^5 $TCID_{50}$ of either mutant or wild-type parental virus (BICv). For screening, pigs were randomly allocated into groups of at least 2 animals each, and pigs in each group were inoculated with one of the virus mutants or BICv. Clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the 21-day experiment. Total and differential white blood cell and platelet counts were

obtained using a Beckman Coulter ACT (Beckman, Coulter, CA). For protection studies, pigs were inoculated with 10^5 $TCID_{50}$ of the corresponding attenuated virus. At 28 days post-inoculation (DPI) animals were intranasally challenged with 10^5 $TCID_{50}$ of BICv. Clinical signs and body temperature were recorded daily throughout the experiment as described above. Blood, serum, nasal swabs and tonsil scrapings were collected at times post-challenge.

Results:

CSFV structural Core protein binds swine SUMO-1, UBC9 and IQGAP host proteins.

To identify possible host cellular proteins that interact with CSFV Core protein, we constructed an N-terminal fusion of the Gal4 protein binding domain to the Core protein as 'bait' for the yeast two-hybrid system. We screened >1 x 10^7 independent yeast colonies, from a swine primary macrophage cDNA library containing 3 x 10^6 independent clones. These colonies were selected for growth on -Leu/-Trp/-His/-Ade media. Plasmids were isolated from positive colonies and sequenced. In-frame proteins were retested for specificity to the Core protein. As a negative control proteins were tested for binding the Lam-BD protein and BD protein alone. Several proteins were determined as specific binding partners for the CSFV Core protein (data not shown). Three of these proteins, SUMO-1, UBC9, and IQGAP were selected for further study. All these proteins were demonstrated to specifically bound Core protein when compared to binding of BD proteins alone (Fig. 2, 3 and 4).

Mapping areas of CSFV Core protein critical for SUMO-1, UBC9 and IQGAP recognition.

Sumoylation generally occurs at specific lysine residues in target proteins harboring the consensus motif ψKxD/E/P/G (where ψ is a bulky hydrophobic residue). Using SUMOplot Analysis Program (www.abgent.com/tools/SUMOplot) we were able to predict sumoylation domains with the consensus sequence ΨKxD/E/P/G within CSFV strain Brescia Core protein (Fig. 2, 3 and 4). Four putative lysine residues at amino acid positions 179, 180, 221 and 246 of the CSFV polyprotein were identified as possible binding sites with SUMO-1 protein. An additional lysine at position 220 was also identified as a possible sumoylation site due to its proximity to residue 221. Each of these five lysine residues was in turn substituted for alanine by site-directed mutagenesis. Individual and combined substitutions produced the following Core protein mutants: CoreΔS179, CoreΔS180, CoreΔS220, CoreΔS221, CoreΔS246, CoreΔS179/180, CoreΔS220/221, and CoreΔS179/180/220/221 (Table 1). Then, the CoreΔS mutated proteins were tested in the yeast two-hybrid system against swine SUMO-1 and UBC9 proteins. Interestingly, only CoreΔS220 lost its ability to bind UBC9 and none of the individual lysine-to-alanine mutants lost the ability to interact with SUMO-1. Moreover, double mutant proteins K179A/K180A and K220A/K221A retained their interacting capabilities with SUMO-1 (Fig. 4). Sequence analysis of the 100 amino acid residues of Core protein from geographically and temporally different CSFV strains revealed a high degree of sequence similarity and high degree of conservancy at putative sumoylation target sites (Fig. 3). Therefore, for fine mapping the interaction and to determine if other unpredicted residues may be responsible for a possible redundancy in the ability of CSFV Core to interact with swine SUMO-1, amino and carboxyl termini of the protein were assayed separately with the yeast two-hybrid system (Fig. 4). Both amino and carboxyl termini halves of Core retained their SUMO-1 binding capabilities. However, truncated double K179A/K180A and single K221A mutants lost that ability (Fig. 4). Hence, K179, K180, and K221 are mediating the interaction and appear to be the target for SUMO-1 binding, whereas K220 mediates UBC9 binding. For IQGAP, comparative analysis was performed based in the previous finding that that Moloney murine leukemia virus (MMLV) matrix (M) protein also binds IQGAP1 protein. Mutational studies defined residues of MMLVM critical for its interaction with IQGAP1 revealing a close correlation between binding and virus replication. Based in the sites of MMLVM protein that recognize IQGAP1 four (I-IV) areas in CSFV Core protein were identified by using the ClustalW program. CSFV mutants harboring substitutions for alanine for the native amino acid sequence in these four areas were

developed to assess these regions as areas important for IQGAP1 binding to the core protein (Fig. 2). Based on that information the mCSFV Core mutant version of the protein were developed: CoreΔIQ.I, CoreΔIQ.II, CoreΔIQ.III, and CoreΔIQ.IV (Fig. XX). These CoreΔIQ mutated proteins were tested in the yeast two-hybrid system against the swine IQGAP1 protein. Interestingly, substitutions altering areas I and III identified as putative IQGAP1 binding sites lost their ability to bind IQGAP1 (Fig. 1 and 2). Where as, substitutions in areas II and IV caused a decrease in the ability of the core protein to bind the swine IQGAP1 protein. All CoreΔIQ mutated proteins maintained their ability to bind swine clathrin in the yeast-two hybrid at similar levels, suggesting that these mutated areas in the CoreΔIQ mutated proteins, are specific areas for IQGAP1 binding.

Development of CSFV mutant viruses

To further evaluate the role of CSFV Core mutants in the biology of the virus, recombinant viruses based on virulent strain Brescia (BICv) were constructed, containing single or combined alanine substitutions in the previously described critical residues of the Core gene. Mutations were introduced by site-directed mutagenesis into a full-length cDNA copy of CSFV strain Brescia (pBIC). Infectious RNA was *in vitro* transcribed from full-length ICs of pBIC or a set of SUMO-1/UBC9 and IQGAP (CoreΔS) mutants (Fig. 2, 4 and 5) for use in SK6 cell transfections. Mutant viruses referred to as CoreΔS179/180/220/221v, CoreΔS179/180v, CoreΔS220/221v, CoreΔS179v, CoreΔS180v, CoreΔS220v, CoreΔS221v and CoreΔS246v represent each of five of the putative SUMO-1 binding sites at lysine residues in positions 179, 180, 220, 221 and 246 of the CSFV polyprotein (Fig. 4). Regarding the IQGAP mutants viruses were referred to as CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, and CoreΔIQ.IVv representing each of four of the putative IQGAP1 binding sites CSFV Core protein (Fig. 2).Viruses were rescued from transfected cells by 4 dpi (days post-infection). Nucleotide sequences of viable rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at predicted mutated sites were reflected in rescued viruses.

Replication of Core mutant viruses *in vitro*

In vitro growth characteristics of all viable Core mutant viruses: CoreΔS179/180/220/221v, CoreΔS179/180v, CoreΔS220/221v CoreΔS179v, CoreΔS180v, CoreΔS220v, CoreΔS221v, CoreΔS246v, and CoreΔIQ.I v to IVv viruses were evaluated relative to parental pBIC-derived virus (BICv) in a single-step growth curve (Fig. 6). Primary swine macrophage cell cultures were infected at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ per cell. Virus was adsorbed for 1 hour (time zero), and samples were collected at 72 hours post-infection (hpi). All mutant viruses exhibited titers similar to parental BICv except CoreΔS179/180/220/221v, with a virus yield approximately 1.5 log10 $TCID_{50}$/ml lower than BICv, and (Fig. 6). This indicates that although none of the introduced mutations individually affected virus titer, combined mutations significantly affected virus yield. Regarding the IQGAP viruses all exhibited about one order of magnitude of decrease in titer when compared with parental BICv suggesting that all viruses have an *in vitro* growth defect when compared to parental BICv (data not shown).

Evaluation of the role of CSFV Core sumoylation sites in CSFV virulence in swine

To examine the effects of deletion of Core sumoylation sites on CSFV virulence, CoreΔSv mutant viruses CoreΔS179/180v, CoreΔS220/221v, CoreΔS220v, CoreΔS221v, CoreΔS246v and CoreΔS179/180/220/221v were intranasally (IN) inoculated into naïve swine, at doses of 10^5 $TCID_{50,}$ and monitored for clinical disease relative to the parental virus. After inoculation, survival of pigs inoculated with mutant viruses was assessed relative to lethal exposure of control animals infected with virulent BICv. BICv exhibited a characteristic virulent phenotype (Table 1): none of the control pigs survived the infection, dying or being euthanized around 9 dpi. Interestingly, mutant CΔS179/180/220/221v was completely attenuated in swine. Animals survived the infection and remained clinically normal throughout

the observation period (21 days). This virus harbors lysine-to-alanine substitutions at positions 179, 180, and 221, three residues that are responsible for Core-SUMO-1 binding, and at position 220 for Core-UBC9 binding. In contrast, only two out of five swine that displayed severe signs of CSF disease survived IN infection with mutant CoreΔS179/180. Conversely, animals infected with CoreΔS220/221v showed no CSFV-related clinical symptoms during the 21-day observational period. Animals infected with mutant CoreΔS220v, although presenting less severe CSF symptoms than those infected with BICv, died around 16 dpi. Remarkably, animals infected with mutant CoreΔS221v also survived the infection, showing only a transitory rise in body temperature around 4 dpi. Animals infected with mutant CΔS246v had normal binding to both SUMO-1 and UBC9 and presented a disease progression indistinguishable from those infected with BICv (Table 1 and Fig. 6). Therefore, there appears to be a close correlation between disrupting Core-SUMO-1 and Core-UBC9 binding as detected in the yeast two-hybrid system and the induction of virus attenuation. Virus shedding (detected in tonsil scrapings and nasal swabs) and viremia in CoreΔS179/180/220/221v and CoreΔS221v-inoculated animals were undetectable (sensitivity of detection $\geq$1.8 $TCID_{50}$/ml) at all time points sampled (Fig. 7). Similar results were obtained in animals infected with CoreΔS220/221v, with the exception of one animal (out of five inoculated) showing transient low virus titers in blood, tonsil scrapings and nasal swabs around 14 dpi. Conversely, animals infected with CoreΔS220v displayed lower titers (1.5 to 3.5 $\log_{10}$ regarding the time point and clinical sample considered) in comparison to animals infected with BICv (Fig. 7). Animals infected with CoreΔS179/180 presented a similar phenotype: titers of intermediate intensity, compared with those in BICv-infected swine, that last until animals die or around 15 dpi. Animals infected with CoreΔS246v present values of virus shedding and viremia indistinguishable from those infected with BICv.

Evaluation of the role of CSFV Core IQGAP1 binding sites in CSFV virulence in swine

To examine the effects of deletion of Core protein interaction with IQGAP1 protein, on CSFV virulence, CoreΔIQGAPv mutant viruses I, II, III and IV were intranasally (IN) inoculated into naïve swine, at doses of 10^5 $TCID_{50,}$ and monitored for clinical disease relative to the parental virus. After inoculation, survival of pigs inoculated with mutant viruses was assessed relative to lethal exposure of control animals infected with virulent BICv. BICv exhibited a characteristic virulent phenotype (Table 2 and Fig 2): none of the control pigs survived the infection, dying or being euthanized around 8 dpi. Interestingly, viruses CΔIQGAP.I and CΔIQGAP.IIIv were completely attenuated in swine. Animals survived the infection and remained clinically normal throughout the observation period (21 days) with only one animal out five infected with CΔIQGAP.III presenting a raise in body temperature for one day. In contrast, animals infected with mutant CΔIQGAP.II or CΔIQGAP.IVv, although presenting less severe CSF symptoms than those infected with BICv, died around 12 and 14 dpi, respectively (Table 2). Therefore, there appears to be a close correlation between disrupting Core-IQGAP1 protein binding as detected in the yeast two-hybrid system and the induction of virus attenuation. Virus shedding (detected in tonsil scrapings and nasal swabs) and viremia in CΔIQGAP.Iv- and CΔIQGAP.IIIv-inoculated animals were almost undetectable (sensitivity of detection $\geq$1.8 $TCID_{50}$/ml) at all time points sampled. Interestingly, animals infected with CΔIQGAP.IIv and CΔIQGAP.IVv presented intermediate titers in both nasal swabs and tonsil scrapping but significantly high titers in blood (data not shown), similar to those seen in BICv infected animal.

Discussion:

We describe for the first time the involvement of CSFV Core protein in virulence of CSFV. We report here the identification of host binding partners Sumo-1, UBC9 for CSFV Core protein. Disruption of Core protein binding to host proteins UBC9 and SUMO-1, and IQAP in the yeast two-hybrid system, present a close correlation with the induction of virus attenuation *in vivo*. This would suggest that the sumoylation pathway plays an important role in CSFV pathogenesis. Two distinct regions in the Core

protein were identified that are required for binding SUMO-1 (K179, K180) and (K221), whereas residue K220 was important for UBC9 binding. Assessment of *in vitro* growth ability of CoreΔS viruses demonstrated that alteration of SUMO-1 or UBC9 binding sites in Core protein produces viruses having similar virus yields to wild-type, with the exception of Core□S1234 that displayed a reduced titer of 1.5 $\log_{10}$ lower. However, *in vivo* defects in replication had a direct correlation with their virulence. It is possible that this defect in replication allows the host immune response to overcome initial CSFV infection. In fact, CoreΔS viruses presenting the highest extent of attenuation demonstrate a severe decreased ability for replication during infection in animals.

Although this is the first time a correlation between the sumoylation pathway and CSFV has been described, other viruses have been shown to hijack the sumoylation pathway to alter host protein sumoylation. Ebola Zaire and Adenovirus infections interfere with the sumoylation pathway, preventing host proteins from being sumoylated [28, 29]. Therefore, it is possible that CSFV Core protein binds SUMO-1 and UBC9 in an effort to evade the host immune response. Although the mutation K220A in Core disrupts the binding of UBC9 in the yeast two-hybrid system, this does not imply that the K220A Core protein cannot be sumoylated at other residues. Other studies have shown that in the yeast two-hybrid system proteins can be sumoylated without the detection of UBC9 binding, suggesting that UBC9 binding may have a role other than directly sumoylating the Core protein. Our results show that mutation K220A partially affected virus pathogenesis, possibly due to lack of UBC9 binding. However, mutating Core residue K221A is critical to virus virulence, producing only a transient rise in body temperature. Furthermore, double mutation K220A/K221A resulted in complete attenuation, suggesting that mutation of K220A only would allow partial SUMO-1 or UBC9 binding, but mutation of both of these residues abrogates binding in this area of the Core protein. Separate mutations of residues K179A and K180A showed no difference in virus virulence when compared to wild-type (data not shown). However, pathogenesis was altered when a double mutation K179A/K180A was introduced, and resulted in a delayed infection and survival of some of the animals. This suggests that this area of the virus also requires binding of SUMO-1.

We performed experiments to analyze alterations in the location of Core protein when putative sumoylation sites where mutated. Nevertheless, we were unable to see differences in the intracellular localization of the wild-type Core protein or the double mutant Core proteins (K179/K180 and K220/K221) when they were overexpressed and tagged with GFP (data not shown). However, the involvement of SUMO-1 in protein localization cannot be excluded. It is possible that the localization of the Core protein could be dependent on sumoylation only when expressed as a polyprotein, or when part of the virus structure, but a Core antibody was not available to test this hypothesis. Western blots performed using an anti GFP monoclonal antibody also could not detect sumoylation of the GFP-Core protein (data not shown). Proteins that are sumoylated usually are detected in small amounts, so it is possible that this overexpression of Core-GFP was able to mask a small percentage of the Core protein that is sumoylated during the virus lifecycle, and may also be dependent on Core being expressed first as a polyprotein, or in the presence of other virus proteins in the correct proportions.

Regarding the binding to the host protein IQGAP, we describe for the first time the involvement of the swine IQGAP protein with CSFV Core protein. Disruption of Core protein binding with IQGAP1 in the yeast two-hybrid system, presents a correlation with the induction of virus attenuation *in vivo*. Whereas, partial disruption of IQGAP1 binding results in a delayed onset of CSF, suggesting that the degree of attenuation closely correlates with the ability of the core protein to interact with IQGAP1, and that this swine host protein interaction plays an important role in CSFV pathogenesis.

Assessment of *in vitro* growth ability of IQGAPΔ viruses demonstrated that alteration of IQGAP1 binding sites in Core protein produces viruses having virus yields approximately one $\log_{10}$ less then wild-type. However, *in vivo* defects in replication had a direct correlation with their virulence. It is possible that this defect in replication allows the host immune response to overcome initial CSFV infection. In fact,

CΔIQGAP,viruses presenting the highest extent of attenuation demonstrate a severe decreased ability for replication during infection in animals.

Although this is the first time IQGAP1 has been described as a virulence factor during CSFV infection, IQGAP1 has been implicated as a virulence factor for MuLV virus infection (REF). Bacterial infections have also implicated IQGAP one as being an important factor during pathogenesis such as in Pseudomanas (19910414) and during salmonella infection (19956712). In addition, IQGAP1 is involved in lymphocyte transendothelial migration (20017185), and tumor evasion (19877006). Suggesting IQGAP1 plays an important role in the pathogenesis of various diseases.

In summary, we identified the first swine host protein binding partners for the CSFV Core protein: SUMO-1, UBC9 and IQGAP. We describe the active binding sites in CSFV Core protein to SUMO-1, UBC9 and IQGAP and it is shown that disruption of these specific binding sites in the Core protein can completely abrogate virus virulence, demonstrating that acquisition of attenuation correlates with loss of binding to components of the sumoylation pathway, SUMO-1, UBC9 and IQGAP. Furthermore, it is necessary to gain additional insight into host-viral protein interactions in order to understand the mechanisms of viral virulence and immune system evasion. Understanding the pathways and the protein players involved is necessary for developing and understanding better countermeasures to control virus infection in swine.

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Table 1: Swine survival and fever response following infection with CSFV Core□S mutants and parental BICv

Virus	No. of survivors/ total no.	Mean time to death (days ± SD)	Fever		
			No. of days to onset (days ± SD)	Duration no. of days (days ± SD)	Max daily temperature (± SD)
CoreΔS1234	2/2	No	No	No	103.3 (0.4)
CoreΔS12	2/5 (1)	15.3 (0.6)	4.4 (1.5)	11.8 (3.4)	106.1 (1)
CoreΔS34	5/5	No	No	No	103.2 (0.4)
CoreΔS3	0/2	16.5 (6.4)	4 (0)	10 (2.8)	106.2 (0.3)
CoreΔS4	2/2	No	4 (2)	4 (2)	104.4 (0.9)
CoreΔS5	0/2	9 (0)	4.5 (0.7)	4.5 (0.7)	105.6 (0.3)
BICv	0/4	9 (0.8)	3.5 (0.6)	5.5 (1)	106.2 (0.9)

(1) The 2 surviving animals showed severe signs of CSF at the end of the experimental period (21 days).

(2) Only one animal showed a transitory increased body temperature

Table 2: Swine survival and fever response following infection with CSFV IQGAP1 mutants and paretal BICv

Virus	No. of survivors/ total no.	Mean time to death (days ± SD)	Fever		
			No. of days to onset (days ± SD)	Duration no. of days (days ± SD)	Max daily temperature (± SD)
IQGAP.I	6/6	No	No	No	102.8 (0.6)
IQGAP.II	0/7	12 (0.5)	5.9 (2.5)	6.2 (2.7)	106.2 (1.1)
IQGAP.III	5/5	No	4.5 (0.7) (1)	1 (0)	103.8 (0.7)
IQGAP.IV	1/5	14 (2.4)	4 (0)	6.8 (3.6)	105 (1.2)
BICv	6/6	8.5 (1.9)	3.5 (0.6)	5 (1.6)	106.2 (0.7)

(1) Only one animal transitorily raised its body temperature

Table 3. Set of CSFV CoreΔSumo mutant viruses constructed in this study.

CSFV polypeptide position	**Wild Type Sequence**	**Mutant Sequence**	**Mutant**
179	KE**K**KPDR	KE**A**KPDR	CoreΔS1
180	KEK**K**PDR	KEK**A**PDR	CoreΔS2
179/180	KE**KK**PDR	KE**AA**PDR	CoreΔS12
220	QVQ**K**KGK	QVQ**A**KGK	CoreΔS3
221	QVQK**K**GK	QVQK**A**GK	CoreΔS4
220/221	QVQ**KK**GK	QVQ**AA**GK	CoreΔS34
246	SRK**K**LEK	SRK**A**LEK	CoreΔS5
179, 180, 220, 221	KE**KK**PDR, QVQ**KK**GK,	KE**AA**PDR, QVQ**AA**GK,	CoreΔS1234

Figure Legends

Fig. 1: Interaction of CSFV Core with swine SUMO1 and UBC9 proteins in yeast two-hybrid system. The CSFV Core protein was fused to the binding domain of Gal4 (BD). Sumo1 and UBC9 are fused to the activation domain of Gal4 (AD). Yeast strain AH109 carrying the indicated combinations of plasmids were spotted on **(A)** SD- Ade/His/Leu/Trp Plates (plasmid selection and protein interaction selection) or **(B)** control plates SD-Leu/Trp (plasmid selection only)

Fig. 2: Reactivity of CSFV Core with swine wild type and mutant IQGAP proteins in yeast two hybrid system. WT and Core mutants I, II, III and IV proteins were tested in their ability to bind IQGAP protein. Schematic representation of putative IQGAP binding motifs found in CSFV Core protein is depicted on the top of the figure.

Fig. 3: Multiple alignments of CSFV Core proteins revealed the presence of highly conserved putative sumoylation (bold, underlined and in italics) and IQGAP binding sites. IQGAP sites (bold letter): Site I (blue), II (green), III (red) and IV (black). Shown here is a Core protein amino acid sequence comparison between CSFV geographically and temporally separated.

Fig. 4: Schematic representation showing the observed binding patterns between wild type and CSFV Core protein mutants with SUMO1 and UBC9 proteins. Depicted are putative SUMO1 and UBC9 binding sites and K to A substitutions in CSFV Core proteins. Numbers indicate amino acid position relative to CSFV strain Brescia polypeptide.

Fig. 5: Schematic representation showing pattern of reactivity of wild type CSFV Core protein and its mutated forms with IQGAP protein. Also, virulence of the CΔIQGAPv compared with the parental wild-type BICv virus is presented. Positions of the putative IQGAP binding sites are depicted as well as their replacements to A. Numbers indicate amino acid position at the CSFV Brescia isolate polypeptide.

Fig. 6: *In vitro* growth characteristics of CoreΔS mutants and parental BICv. Primary swine macrophage cell cultures were infected (MOI= 0.01) with each of the Core mutants or BICv and virus progeny yield titrated at 72hs post infection in SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: $\geq$ log 10 1.8 TCID50/ml.

Fig. 7*:* Virus titers in clinical samples (blood, tonsil scrapings, and nasal swabs) from pigs infected with CoreΔS mutants and parental BICv. Each point represents the mean log10 TCID50/ml and standard deviations from at least two animals. Sensitivity of virus detection: $\geq$ log 10 1.8 TCID50/ml.

Fig. 1

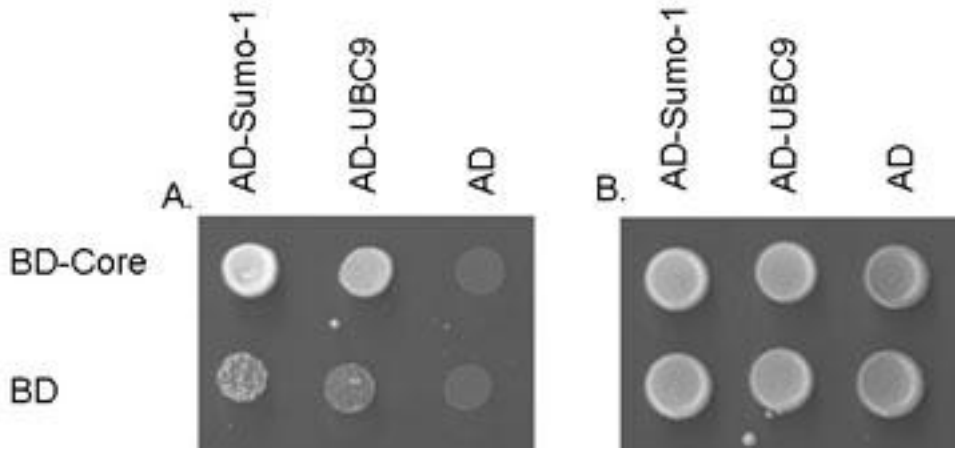

Fig. 2

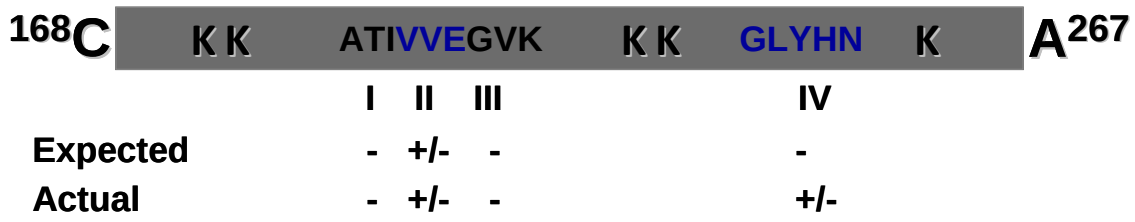

Fig. 2

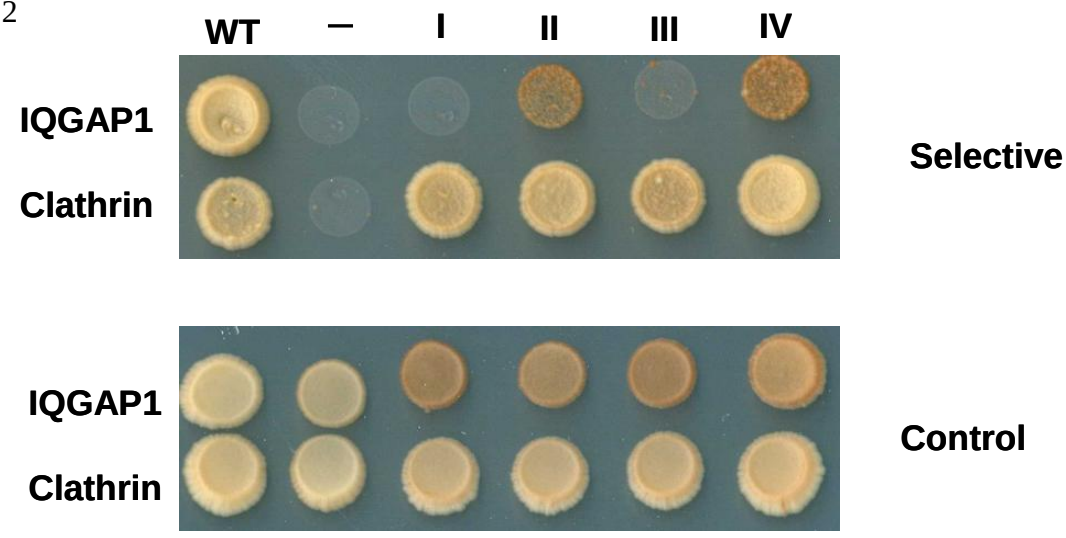

Fig. 3

```
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CSDDGASASKEKKPDRINKGKLKIAPKEHEKDSRTKPPDATIVVEGVKYQVKKKGKVKGKNTQDGLYHNKNKPPESRKKLEKALLAWAVIAIMLYQPVAA

Alfort 187 X87939
.......G.....................................................................E.
LOM EU789580
.......G.....................................................................E.
cF114 AF333000
.......G.....................................................................E.
Alfort A19 U90951
.......G.....................................................................E.
GPE- D49533
.......G.....................................................................E.
Shimen AY775178
.......G.....................................................T.......E.
JL1(06)EU497410
.......G.....................................................T.......E.
SWH DQ127910
.......G.....................................................T.......E.
Thiverval EU490425
.......G..........................................R..............T.............E.
Eystrup NC002657
.......G..........................................S..........................E.
ALD D49532
.......G..........................................S..........................E.
CAP X96550
.......G..........................................R..............V.....E.
Glentorf U45478
.......G..........................................R..............V.....E.
HCLV AF531433
.......G.........................R.....................................E.
Strain C AY805221
.......G.........................R.....................................E.
Riems AY259122
.......G.........................R.....................................E.
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CS AY578688
.......G.............T.....................................................................
GXWZ02 AY367767
......GG..D.....M.............K.....................................................VT.V.......
virus 39 AF407339
.......G..D.....M.............K..S.................................R...................V..V.......
Spain01 FJ265020
.......GN.D.....M.............K.............I.....................................V.......
96TD AY554397
....S.GG..D.....MS............K.................N.V.................................T.V.......
0406 Taiwan AY568569
......GG..D.....M.............K.............................................K...........V.....V.
Paderborn AY072924
......GG.E......M.............K.....................................................T.V.......
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Fig. 4

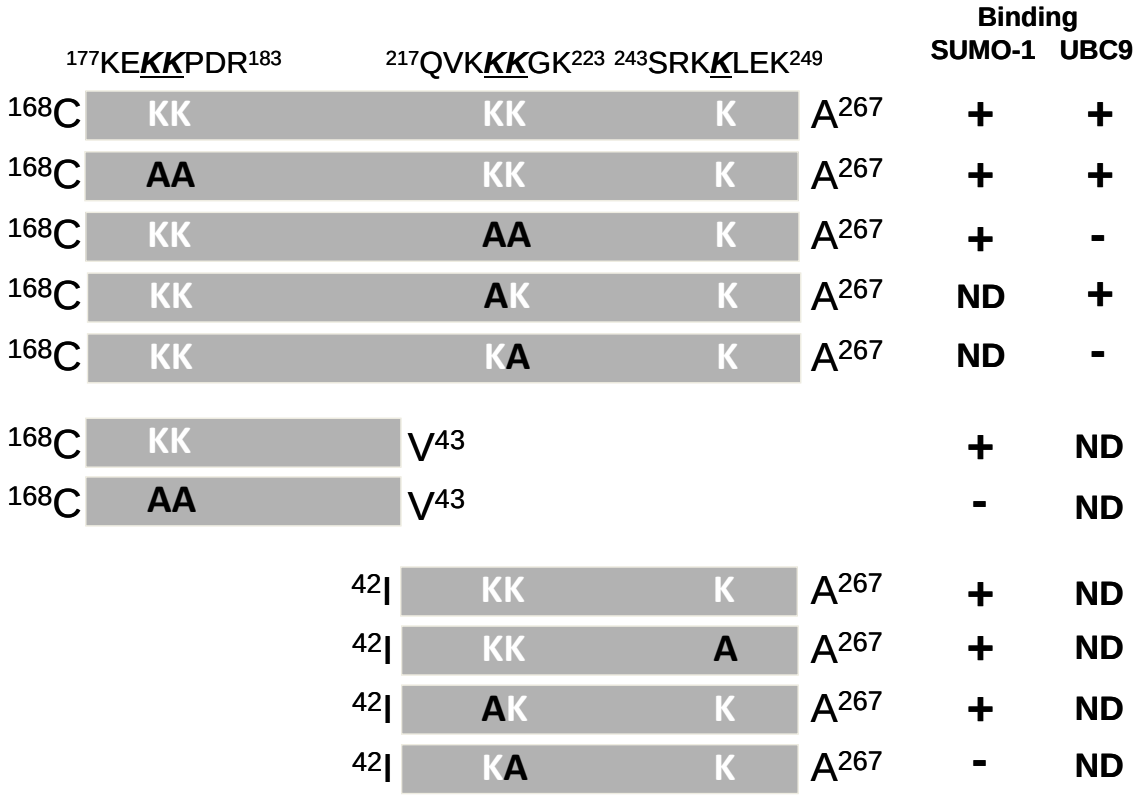

Binding
SUMO-1 UBC9
177KEKKPDR183
217QVKKKGK223 243SRKKLEK249
168C KK KK K A267 + +
168C AA KK K A267 + +
168C KK AA K A267 + -
168C KK AK K A267 ND +
168C KK KA K A267 ND -
168C KK V43 + ND
168C AA V43 - ND
42I KK K A267 + ND
42I KK A A267 + ND
42I AK K A267 + ND
42I KA K A267 - ND

Fig. 5

CORE	IQGAP binding	Animal Survival
WT	+++++	No
IQGAP1	–	Yes
IQGAP2	+	delayed
IQGAP3	–	Yes
IQGAP4	++	delayed

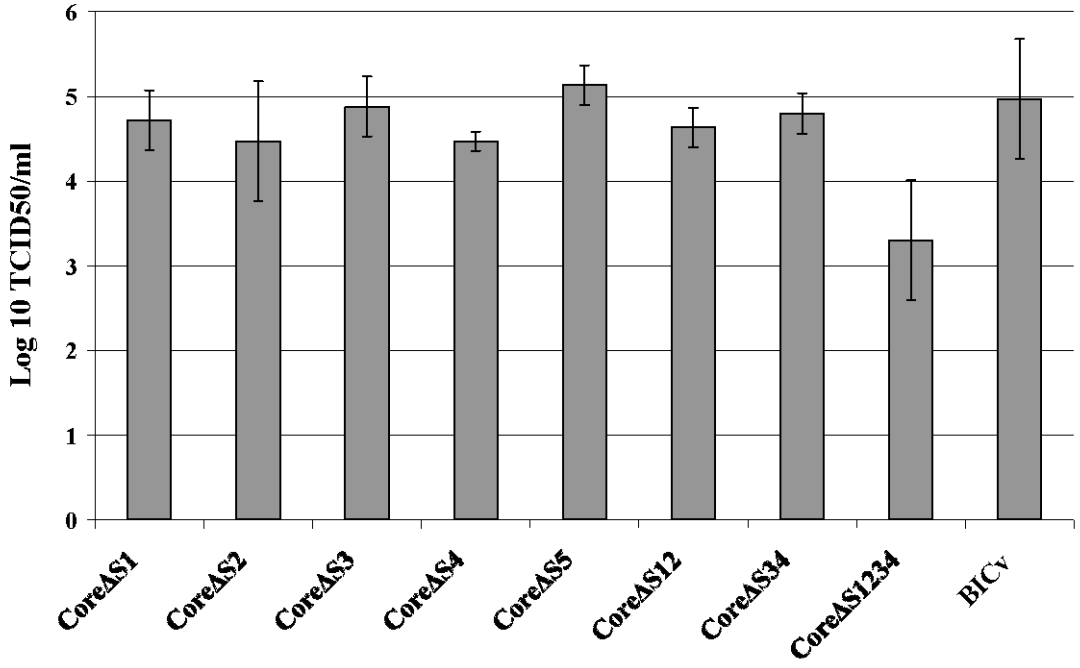
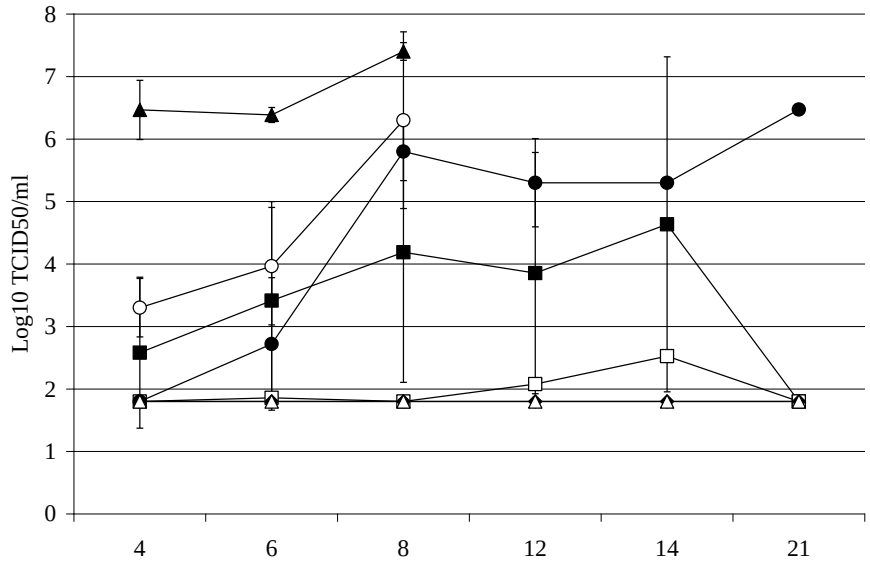

Log 10 TCID50/ml
6
5
4
3
2
1
0
CoreΔS1
CoreΔS2
CoreΔS3
CoreΔS4
CoreΔS5
CoreΔS12
CoreΔS34
CoreΔS1234
BICv

Fig. 6

Fig. 7

A


Log10 TCID50/ml
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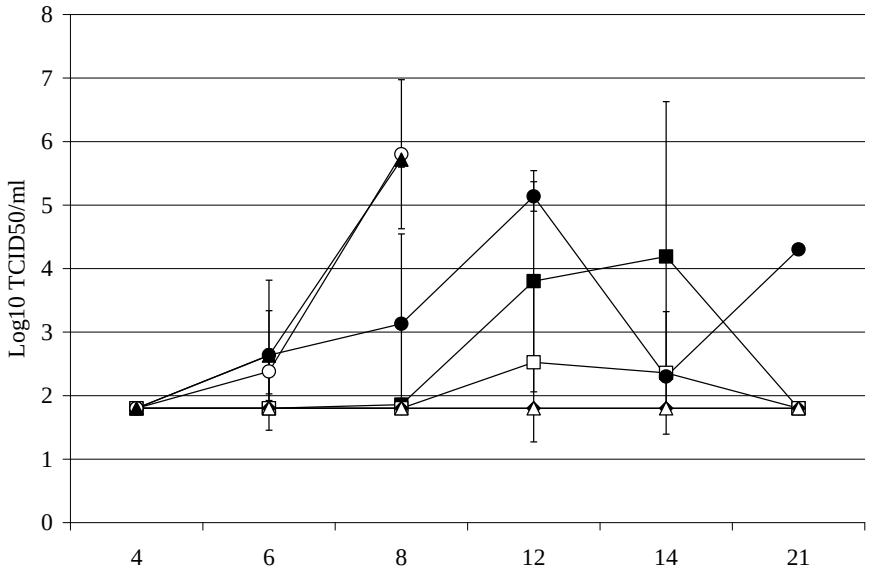
1
2
3
4
5
6
7
8
4
6
8
12
14
21

B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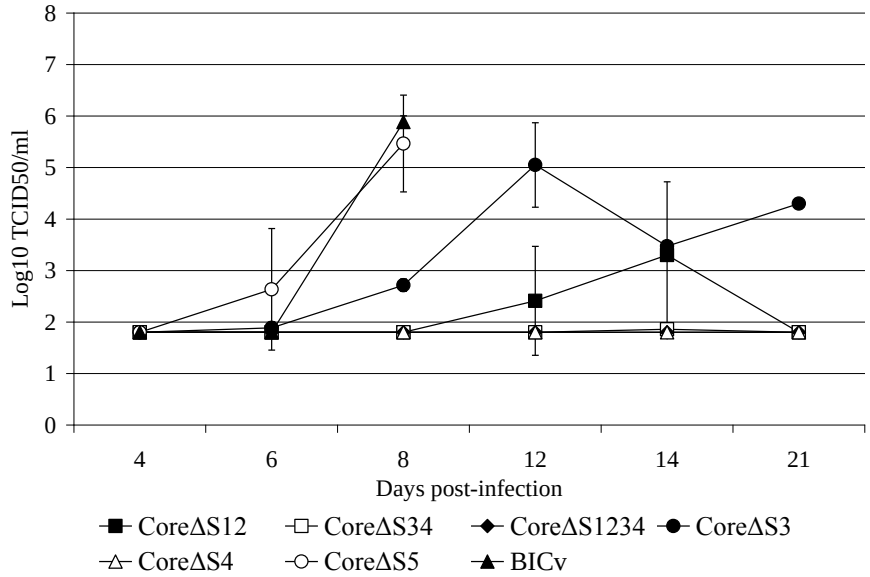
1
2
3
4
5
6
7
8
4
6
8
12
14
21

C


Log10 TCID50/ml
0
1
2
3
4
5
6
7
8
4
6
8
12
14
21
Days post-infection
CoreΔS12
CoreΔS34
CoreΔS1234
CoreΔS3
CoreΔS4
CoreΔS5
BICv