# CLONING AND EXPRESSION OF FECV SPIKE GENE IN VACCINIA VIRUS

## Immunization with FECV S Causes Early Death after FIPV Challenge

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## ABSTRACT

The spike gene of the feline enteric coronavirus (FECV), strain FECV-1683, was PCR amplified from total RNA extracted from FECV-infected cells and its sequence determined. A primary translation product of 1454 amino acids is predicted from the nucleotide sequence, containing a N-terminal signal sequence, a C-terminal transmembrane region and 33 potential N-glycosylation sites. The sequence shares 92% homology with the previously published feline infectious peritonitis virus, strain WSU-1146; however, several regions were identified that distinguished FECV from Feline Infectious Peritonitis virus, FIPV. The full length FECV S gene was cloned and expressed in vaccinia virus. Recombinants produced a 200 kD protein which was recognized by sera from cats infected with FIPV. When kittens were immunized with the vaccinia/FECV S recombinant, neutralizing antibodies to FIPV were induced. After challenge with a lethal dose of FIPV, the recombinant vaccinated animals died earlier than control animals immunized with vaccinia virus alone.

## INTRODUCTION

Two laboratory strains of feline coronavirus, feline infectious peritonitis virus (FIPV WSU 79-1146 and a closely related Nor15/DF2) and feline enteric coronavirus FECV 1683 have been studied extensively in the literature [1-5]. WSU 79-1146, although antigenically similar to FECV 1683 exhibits a different type of pathogenesis. The hallmark of FIPV

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infection is an immune-mediated peritonitis which can be accelerated experimentally by previous exposure to the virus [2]. Cats with FIP disease typically have high circulating antibody titers as well as deposition of antigen-antibody complexes in many major organs. Susceptibility of macrophages/monocytes to virus infection is believed key to the widespread immunopathology observed following FIPV infection[6].

The spike protein appears to be largely the focus of the immune mediated pathology in that the other major structure proteins (nucleocapsid and membrane) cannot induce sensitization to early death syndrome [7]. Vennema et al., reported that a lethal FIPV challenge of kittens immunized with a vaccinia recombinant expressing the FIPV S protein developed FIP disease and died more rapidly than non-vaccinated animals following virulent FIPV challenge [8] More recently, Horsburgh et al., [9] showed that immunization with vaccinia recombinants expressing either the CCV or TGEV S protein accelerated the death of kittens challenged with a lethal dose of FIPV indicating a conserved feature of the spike protein immune response that predisposes cats to FIP.

The differences between FIPV-like and FECV-like virus may not be do to the genetic specificity of the virus, but rather, to the site of replication. Because the cell tropism of FECV 1683 differs dramatically from FIPV, the sequence of the FECV (1683) spike gene was determined and compared to that of the FIPV WSU 79-1146 and Nor15/DF2. FECV 1683 does not cause accelerated death nor FIP symptoms upon oral/nasal exposure of the virus to 12 week old kittens, in contrast Nor15/DF2 can cause acute symptoms with only a single exposure when administered by this route. To examine whether FECV spike protein retains the specificity of immune response pathology as its antigenically similar sister FIPV-DF2 the spike proteins of both were expressed using vaccinia virus vectors and compared side by side in a cat immunization study[7].

## MATERIALS AND METHODS

*Materials.* All restriction enzymes were purchased from New England Biolabs (Beverly, MA) or Bethesda Research Labs (Gaithersburg, MD) and used according to manufacturer's specifications. Bluo-Gal was obtained from Sigma. All PCR reagents were produced by Perkin Elmer-Cetus (Norwalk, CT). Alkaline-phosphatase labeled antibodies and the BCIP/NBT substrate system were received from Kirkegaard-Perry Laboratories, Inc. Anti-FIPV serum was produced at SmithKline Beecham Animal Health (SBAH), Lincoln, Nebraska in cats that were vaccinated with a temperature sensitive DF2 strain of FIPV (Primucell[R] SmithKline Beecham Animal Health) and challenged with DF2 FIPV [10]. POTSKF33 was obtained from Dr. Christine DeBouck, SB Pharmaceuticals.

*Cells and Virus Strains* Norden Laboratories Feline Kidney (NLFK) cells and Type II FIPV Nor15/DF2 were grown and maintained as described previously [10,11]. A feline enteric coronavirus, FECV (WSU-1683), was obtained from Washington State University. Vaccinia virus strain WR was received from Dr. Bernard Moss, NIH.

For vaccinia virus infections, human thymidine-kinase-negative (HuTK-) and African green monkey kidney (CV-1) cell lines were used and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum.

*RNA purification.* Roller bottles of confluent NLFK cells were infected with either DF2 FIPV or FECV virus at MOI = 0.1 in 50 ml of BME supplemented with 2% FBS. DF2 FIPV infections were performed in serum-free medium. The virus was absorbed for 2 hours and then 250 ml of growth medium added. The cultures were monitored for cytopathic effect (CPE) and typically harvested at 24 - 36 hours post-infection. Total cytoplasmic RNA was prepared from the infected monolayers by guanidine isothiocyanate extraction [12]

*Oligonucleotide Design and Synthesis.* Oligonucleotides were designed based on the nucleotide designation of the WSU 1146 FIPV S gene (GenBank Accession # X06170) using *Sma* I and *Stu* I sequences at the ends to facilitate cloning into pSC11. The primers were synthesized on an Applied Biosystem Model 380B DNA Synthesizer using the phosphoramidite method and were gel-purified prior to use.

*PCR Amplification.* Synthesis of cDNA from total RNA isolated from cells infected with a specific coronavirus was performed using standard procedures. Amplification of the cDNA was performed essentially according to the method of Saiki *et al.*, [12] using the *Taq* polymerase. The reaction was performed in the Perkin-Elmer Cetus thermal cycler for one cycle by denaturing at 95°C for 1', annealing at 37°C for 2' followed by an extension at 72°C for 40'. A standard PCR profile was then performed by a 95°C-1' denaturation, 37°C-2' annealing, 72°C-3' extension for 40 cycles. A final extension cycle was done by 95°C-1' denaturation, 37°C-2' annealing, 72°C-15' extension and held at 4°C until analyzed. PCR products were analyzed by electrophoresing 5.0 µl of the reaction on a 1.2 % agarose gel run 16-17 hours. Bands were visualized by ethidium bromide staining the gel and fluorescence by UV irradiation at 256 nm.

*Cloning and sequencing of the FECV S gene.* DNA sequence was determined from overlapping cloned regions of the FECV S gene. PCR-amplified DNAs were digested with *Xma*I and *Stu*I, excised and eluted from low-melting temperature gels and ligated into pBluescript vector (Stratagene). Insert-bearing clones were identified by restriction mapping. Nested set deletions were prepared and the sequence determined by Lark Sequencing Technologies, Houston TX, from both strands using the chain termination method [14]. DNA sequence analysis was performed using the University of Wisconsin GCG package of programs.

*Cloning of Full-length FIPV and FECV S genes in pSC11.* The full-length FECV S gene was amplified by PCR, digested with *Sma*I and *Stu*I, excised as above amd ligated into a bacterial expression plasmid, pOTSKF33, using standard procedures. Full-length DF2 FIPV and FECV spike gene 1-1454aa inserts were isolated from established pOTSKF33 plasmid clones by *Sma*I/*Stu*I digestion of plasmid DNA and the excised gene cloned into the *Sma*I site of the vaccinia recombination plasmid, pSC11.

*Western Blots of virus expressed proteins.* HuTK- monolayers infected with recombinant viruses were harvested at 2 days post-infection by scraping into the medium. Pelleted cells were washed with PBS and resuspended in 0.6 ml RIPA buffer (0.15 M NaCl, 0.1% SDS, 1.0% Na deoxycholate, 1.0% Triton X-100, 5 mM EDTA, 20 mM Tris, pH 7.4). RIPA lysates were frozen/thawed 3X and sonicated briefly. Non-reducing sample buffer (2% SDS, 80 mM Tris, pH 6.8, 10% glycerol, 0.02% bromophenol blue) was added and samples boiled 10 min prior to electrophoresis on 10% SDS-polyacrylamide gels as described by Laemmli. Proteins were transferred to Immobilon-P (Millipore) at 20-25 mA for 18 h in Tris/Glycine/Methanol buffer. Filters were blocked in 2% skim milk, 1% gelatin, and TBS (20 mM Tris, pH 7.5, 500 mM NaCl) for 1-2 hours (h) at room temperature (RT), rinsed with TTBS (TBS + 0.05% Tween-20) and incubated with anti-FIPV cat serum (from cats vaccinated twice with Primucell$^R$ licensed temperature sensative DF2 FIPV vaccine) at a 1:50 dilution in TTBS and 1% gelatin for 1-2 h at RT. Filters were washed in TTBS 3X for 10 min each and incubated with goat anti-cat alkaline-phosphatase labeled IgG at a 1:1000 dilution for 1 h. Filters were washed as before and incubated 5 - 15 min in BCIP/NBT substrate according to the manufacturer's instructions. Filters were then rinsed in water and air-dried.

## RESULTS

The sequence comparison of the FECV 1683 spike gene (Genbank accession number) and WSU 79-1146 (accession number) are shown in Table 1. The gene is 4365 bp in length

**Table 1.** Percent homology comparison between FECV 1683 and WSU 1146 Comparison values represent percent homology

| Strain | WSU 1146 | DF2 |
|---|---|---|
| Nucleotide comparison | | |
| FECV 1683 | 94 3(255) | 94 3(250) |
| WSU 1146 | | 99 8(15) |
| Amino Acid Comparison | | |
| FECV 1683 | 97 3 (s) | 97 2(s) |
| | 95 5 (i) (67) | 95 4 (i) (67) |
| WSU 1146 | | 99 6 (s) |
| | | 99 6 (i) (8) |

Numbers in parentheses denote actual number of nucleotide or amino acid differences (s) represents percent amino acid homology based on Dayhoff similarity matrix (i) represents percent amino acid identity Predicted number of glycosylation sites for each amino acid sequence are FECV 1683=33 WSU 1146=35 DF2=35

and encodes an open reading frame of 1454 aa (~161,160 d molecular weight) As predicted for a surface glycoprotein, the FECV S gene contained a signal sequence of 14 residues at the amino terminus, a C-terminal transmembrane domain upstream of a 38 amino acid cytoplasmic tail and 33 predicted N-glycosylation sites

The the S gene of FECV-1683 and the WSU-1146 strain share 94 3% homology at the nucleotide level and 95 5% homology at the amino acid level Interestingly, the S gene of FECV is more closely related to CCV at the gene sequence than to FIPV The FECV S gene sequence contains 6 extra nucleotides (positions 351 - 356) when compared to the published WSU-1146 FIPV S gene sequence These two additional amino acids at positions 119 and 120 increase the size of the FECV S gene to 1454 aa in contrast to the 1452 aa reported for WSU-1146 Overall, only 66 amino acid differences were observed between the FECV and WSU-1146 FIPV S sequences (95 5% identity) The greatest area of heterogeneity is in the first 300 amino acids of the N terminal reagion, 89 6% identity, while in the remainder of the gene the homology increased to 97 1%

A recombinant vaccinia virus expressing the full length FECV S gene was generated using standard transfection procedures [15] As a control, a recombinant containing the full length S gene from WT FIPV virus, strain Nor15/DF2, was also constructed The presence of coronavirus DNA in each recombinant was confirmed by hybridization Expression of the spike gene in each recombinant was regulated by the 7 5K early-late promoter such that S protein would be produced throughout the vaccinia replication cycle Lysates prepared from cells infected with the vaccinia recombinants were probed for the production of the spike protein by Western analysis using polyclonal sera to FIPV virus (Figure 1)

Bands of 180-~200 kD were detected in lysates prepared from vaccinia/FECV or FIPV infected lysates which co-migrated with spike protein in FIPV-infected cells These bands were not observed in uninfected cells or in cultures infected with vaccinia alone Both the size and the diffuse nature of the bands suggest that the FECV spike is glycosylated in this expression system

*Immunization of Kittens* 14-week-old SPF kittens, eight per group, were immunized subcutaneously with 2 X $10^7$ PFU of the vaccinia recombinants expressing feline coronavirus

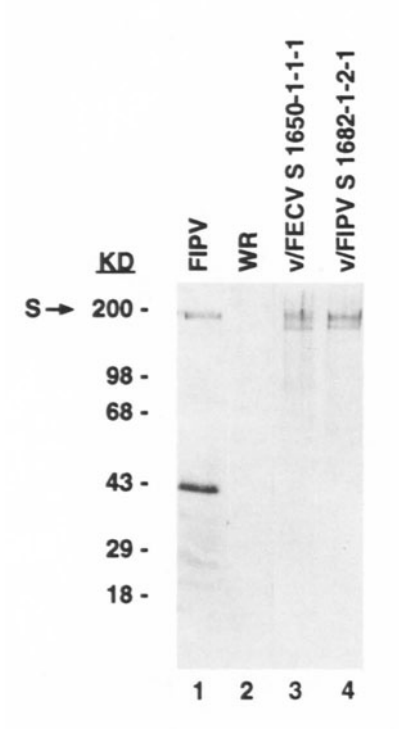


**Figure 1.** Western blots of recombinant S expressed genes. Lane 1 is purified DF2 virus, lane 2 is wt WR strain of vaccinia, lane 3 is wtWR expressing FECV 1683 spike protein, and lane 4 is wtWR expressing FIPV-DF2 spike protein. FIPV cat sera was used for the blot as described in Materials and Methods.

S genes. A group of eight kittens received the same amount of wild-type WR vaccinia virus (v/WR) as a negative control. Kittens were clinically examined; daily and rectal temperatures were taken. A second immunization with the same amount of virus was given after 3 weeks. Two weeks after the second immunization, kittens were challenged orally with 1 X $10^6$ or 1 X $10^4$ PFU of WT DF2 FIPV and survival monitored. Virus-neutralization titers were determined on the day of challenge and one and two weeks post-challenge. Serum samples were taken on the days of first and second vaccination, challenge, and post-challenge days 3, 7, 14, 21, and 28.

The kittens appeared uninfected by the immunization regimen and showed modest seroconversion to both vaccinia and to FECV by ELISA after the second immunization. Virus neutralizing titers were also present at 5 weeks post-vaccination (Table 2). Two weeks after the second immunization, kittens were challenged orally with either 1 X $10^6$ PFU (high dose) or 1 X $10^4$ PFU (low dose) of wild-type (WT) DF2 FIPV and survival monitored. A group of non-vaccinated kittens were also challenged with the low dose of WT DF2 FIPV to serve as infection controls. FIP disease progressed normally in this control group of animals with the first symptoms displayed at 17 -20 days post challenge and a 60% group survival rate after 27 days.

No cats were protected from WT DF2 FIPV challenge regardless of the immunogen but accelerated death was observed as compared to the non-vaccinated controls in groups immunized with vaccinia recombinants expressing either FECV S or DF2 FIPV S. Symptoms of FIP disease were first observed 9 - 11 days after challenge in the animals immunized with the vaccinia/coronavirus recombinants, regardless of the dose of challenge. 100% mortality was observed in the recombinant vaccinated groups as compared with in the non-immunized, challenge controls and in the group immunized with vaccinia alone and challenged with the low dose of WT DF2 FIPV, respectively. Post-mortem examinations were performed on all animals to confirm the diagnosis of FIP.

**Table 2.** Mortality and ELISA antibody titers of vaccinia virus immunized FIPV-challenged kittens

| Vaccine virus | 50% Mortality (days) | Titers by ELISA | | |
|---|---|---|---|---|
| | | 0 PCD | 7 PCD | 14 PCD |
| wtWR low | 27 | 8 | 2,195 | 8,240 |
| wtWR high | 21 | 7 | 2,426 | 14,380 |
| DF2-S low | 18 | 55 | 4,963 | 48,441 |
| DF2-S high | 15 | 84 | 10,149 | 65,554 |
| FECV-S low | 15 | 42 | 3,185 | 56,661 |
| FECV-S-high | 14 | 53 | 1,775 | 11,990 |
| Naive low | 23 | 8 | 754 | 15,875 |

Groups challenged with $10^4$ (low) and $10^6$ (high) dose of virus is indicated Post-challenge days (PCD) Mortality is reported as the number of days to 50% deaths per group IgG serum antibody titers to DF2 FIPV were determined by ELISA, Virus neutralization titers were also measured using conditions previously described [10]

## DISCUSSION

The FECV 1683 spike protein although isolated from a mildly pathogenic virus, shares a high degree of homology with the WSU 79-1146 FIPV spike protein, and can equally sensitize kittens to FIP disease when expressed in the systemic compartment. However, the low incidence of naturally occuringFIP (0.1% in single cat households and 2-5% in multiple cat households), yet high number of cats that are seropositive to coronavirus argues against FECV as a predominant factor in causing FIP in the field. The low incidence of disease must be attributed to more than the spike protein itself since spike gene sequences from CCV, TGE and FECV have now been shown to cause accelerated death in cats. This fact coupled with the high seroprevalence of coronavirus antibodies in cats would indicate that the several other factors separately or acting together cooperate to precipitate disease. In the intestinal tract the virus is evidently innocous, but allowed to replicate systemically (in macrophages) may initiate the disease pathogenesis. The macrophage is the target cell for FIPV infection and dissemination but interestingly, FECV replicates poorly in the macrophage 6. Perhaps vaccinia, which can readily infect macrophages, delivers the FECV spike protein which causes the cascade of immune response pathology induced by the spike protein expressed in this cell type. Under this scenario the mechanism by which the virus can leave the intestinal compartment and infect macrophages may be a worthy area of future study.

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