Comparative sequence analysis of full-length genome of FIPV at different tissue passage levels

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Abstract Feline infectious peritonitis virus (FIPV), an alpha Coronavirus, is the causative agent of a fatal immune mediated disease in cats. It is currently unclear if this virus circulates in the field or develops in felines that are infected with Feline enteric coronavirus. To better understand the genomic changes associated with viral adaptation, we sequenced the complete genomes of FIPV WSU 79-1146 at different tissue passage levels: passage 1, passage 8, and passage 50 tissue culture. Twenty-one amino acid differences were observed in the polyprotein 1a/ab between the different passages. Only one residue change was observed in the spike glycoprotein, which reverted back on subsequent passages, four changes were observed in the 3c protein, and one change was observed in each 3a, small membrane, nucleocapsid and 7a proteins. The mutation rate was calculated to be 5.08–6.3 \times 10^{-6} \text{ nucleotides/site/passage} in tissue culture suggesting a relatively stable virus. Our data show that FIPV has a low mutation rate as it is passed in cell culture but has the capacity for change specifically in nsp 2, 3c, and 7b as it is passed in cell culture.

Keywords Feline infectious peritonitis virus · Feline enteric coronavirus · Pathogenicity · Adaptation

Introduction

Belonging to the family Coronaviridae and the order Nidovirales, Coronaviruses are classified into three main subgroups based on genetic and antigenic differences. Classified in-group alpha, Feline coronavirus (FCoV) infection is common in areas with dense cat populations. FCoV can be divided into two groups: type I and type II. Type I FCoV is considered the “wholly” feline virus, while type II has been identified as a recombinant of Canine Coronavirus (CCoV) and type I FCoV [1]. Within each of these subgroups (type I and II), there are two variants: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV), which cause different forms of clinical disease. The name “FCoV” is typically applied to both. The major difference between the two variants is the clinical presentation and outcome of the disease. The majority of infections with FECV result in a mild gastrointestinal disease, whereas infections with FIPV result in a fatal immune system disease due to serosal membrane damage and widespread pyogranulomatous [2]. While FIPV predominantly affects domestic cats (Felis domesticus), it has been recognized in other species such as the African lion (Panthera leo), the mountain lion (Panthera concolor), leopard (Panthera pardus), and cheetah (Acinonyx jubatus) [3]. There is no accurate diagnostic test for differentiating FIPV from the nonlethal FECV.

In 1996, Poland and colleagues [4, 5] suggested that cats acquire FIPV by a mutation that occurs in an endogenous FECV. This is known as the “in vivo mutation transition hypothesis” which is defined by the FECV disseminating from the gut and going systemic via de novo virus mutations [2, 5]. The conversion from FECV to FIPV is thought to occur more frequently in a primary infection and in kittens <2 years of age [5]. Based on this theory,
several studies have attempted to identify the exact change(s) in FECV that enable the switch in its pathobiology. One study found that the 3c gene, which encodes a small protein of unknown function, was truncated in the majority of FIPV isolates [6]. Although 3c displays more genetic differences in virus isolated from cats with FIPV when compared to 3c in FECV virus isolates, the changes were not shown to be a specific determinant of replication in macrophages [6]. Based on sequence differences, the spike (S) protein as well as the protein encoded by open reading frame (ORF) 7B have also been suggested to play a role in the pathogenicity change but these proteins have not been specifically implicated in the pathobiology of the virus [7]. Although, gene comparisons between FECV and FIPV have identified nucleotide differences, the specific mutations that cause the altered pathotype have not been identified. In addition, the mutations associated with the altered pathotype can even differ between affected kittens in the same litter [6].

A second hypothesis is that FECV and FIPV are two circulating similar viruses that cause different diseases. One study looked at four regions within the genome: the spike-3a–3c (1,017 bp), Membrane (575 bp), Polymerase (384 bp), and 7b (735 bp) in order to demonstrate distinctive circulating virulent and avirulent strains in natural populations [2]. Based on phylogenetic analysis, they concluded that there are clear genetic differences between FIPV and FECV in multiple gene segments. Based on that data the authors concluded that cats are re-infected with new strains of FCoV from external sources, rather than the viruses acquiring mutations in vivo [2]. It is possible that both hypotheses contribute to the manifestation of FIP.

Feline enteric coronavirus have been shown to replicate in mature intestinal epithelium as well as macrophages; however, FIPV isolates have the ability to replicate more efficiently in macrophages ultimately producing a systemic infection that is indicative of FIP [8]. In addition, the carboxy terminal domain of the S glycoprotein of FIPV was shown to be responsible for more efficient macrophage infection [9]. In that study, two genetically engineered FIPVs were constructed with one containing a chimeric S glycoprotein with the N-terminal 873 amino acids derived from FECV and the C-terminal 582 amino acids from FIPV and the other virus containing the N terminal amino acids from FIPV and C-terminal amino acids from FECV [9]. Both recombinants had similar growth kinetics; however, the virus with the C-terminal portion from FIPV was able to readily infect macrophages, whereas the virus with the FECV C-terminal S protein had a reduced ability to infect macrophages with <2 % of the cells infected at 10 h post infection [9]. A more recent study showed that FECV has an intestinal phase and a systemic phase, which involves replication in macrophages [10].

To better understand the capacity of FIPV to genetically change, we examined the full-length viral genome, identifying changes that occurred as the virus was passaged in tissue culture. To evaluate adaption, growth kinetics at each passage level was examined and mutation rates were calculated. Identifying the capacity of FIPV to accommodate genetic changes as it replicates is important for our understanding of the mechanism behind FCoV pathogenesis.

Materials and methods

Cells and viruses

The three viruses sequenced in this study were: FCoV/FIPV/WSU 79-1146 TC passage 1, FCoV/FIPV/79-1146 TC passage 8, and FCoV/FIPV/79-1146 TC passage 50 (Accession numbers: KC461235, KC461236, and KC461237, respectively). All viruses were obtained from ATCC (Manassas, VA 2010). Canine kidney cells (A-72, ATCC CRL-1542) were graciously provided by Dr. Saliki at the Athens Diagnostic Laboratory (University of Georgia). Cells were maintained in Dulbecco’s modified eagles medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). Cells were then cultured in a 37 °C incubator in 5 % CO₂. Working stocks of virus were produced in A72 cells and quantified by plaque assay.

Viral RNA extraction and RT-PCR

Viral RNA was purified using the High Pure RNA Isolation Kit according to the manufacturer’s recommendation (Roche Diagnostic Corporation, Foster City, CA, USA) and re-suspended in DEPC-treated water. Specific primers were synthesized (IDT) for RT-PCR amplification and sequencing based on the published FCoV sequences in Genebank (www.ncbi.nlm.nih.gov). The PCR products were sequenced in both directions using the ABI Prism BigDye Terminator v3.0 (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were analyzed on an ABI 3730 (Applied Biosystems).

Alignment and nonstructural protein comparison

Chromatogram files and trace data for FCoV/FIPV/WSU 79-1146 TC passage levels 1, 8, and 50 were read and assembled using SeqMan Pro, and genome annotation was conducted with SeqBuilder (DNASTAR, Inc., v.8.0.2, Madison, WI, USA). The full-length genome sequence of FCoV/FIPV/WSU 79-1146 passage 100 was previously reported and included in the analysis [11].
Virus titration and viral growth curves

Viruses were grown in A72 cells (passed one time before used for infection) and infected at a multiplicity of infection of 0.1. Growth curves of the isolates examined in this study were performed in A72 kidney canine cells. The culture supernatant was harvested every 12 h after inoculation. To investigate the viral titer in the cells at harvest time, the cells were frozen and thawed three times then filtered. The viral titers were determined by plaque assay. Monolayers were checked daily for identification of plaques. All dilutions for every time point were performed in triplicate.

Molecular evolutionary rate

Mutation rates were calculated by aligning the full-length genomes in Megalign (DNASTAR, Inc., Madison, WI, USA) using the Clustal W algorithm. The distance matrix was calculated for each sequence compared to FCoV/FIPV/WSU 79-1146 TC passage 1, consensus sequence to determine the number of nucleotide changes between the different tissue passages. The sequence differences were then examined in a scatter plot using Microsoft Excel. For comparison purposes, we also calculated the evolutionary rate. In order to estimate the evolutionary rate of the FIPV isolates as they were passed in tissue culture, we used the Bayesian evolutionary analysis sampling trees (BEAST), a program for Bayesian Markov Chain Monte Carlo analysis of molecular data (BEAST v1.4; A. Drummond and A. Rambaut [http://evolve.zoo.ox.ac.uk/]). The evolutionary rate was calculated using a strict clock model and analyzed with Tracer (A. Rambaut and A. J. Drummond [http://evolve.zoo.ox.ac.uk/software.html?id=tracer]) with a discarded burn-in of 10%.

Results

Genomic sequence of FCoV WSU 79-1146

The genomes for all three viruses sequenced in this study were found to be 99.9% similar at the nucleotide level. The genome order was found to be consistent with previous findings 5'-UTR- 1a/1ab-S-3a-3b-3c-E-M-N-7a-7b-3'-UTR [11]. The viruses at all passage levels had the same genome length of 29,357 nt.

ORFs and expression products

The polyproteins 1a and 1ab had 21 amino acid sites that had differences between the viruses examined in this study (Table 1). No amino acid differences were detected in nonstructural proteins 1, 11, 15, and 16. Amino acid changes that were maintained to pass 100 were found in nsp 2 (D177G, W388C, and K807T), nsp 3 (A1784 V), nsp 4 (M2888 K), nsp 6 (A3280 V), nsp 10 (Q3992R), nsp 13 (D5050 V), and nsp 14 (Y5846H). All other amino acid changes in the nsp5 of polyproteins 1a and 1ab reverted back to the Pass 1 virus.

Structural and accessory proteins

The Spike protein is 1,452 amino acids in length. Between pass 0 and pass 8 one amino acid change (T599A) occurred which reverted back to T by pass 50 and was maintained in pass 100 (Table 2). Two other changes were observed between pass 50 and pass 100 (A743V and R1325E).

The open reading frame for accessory protein 3a had one amino acid change between pass 0 and pass 8 one amino acid change (F61S) which reverted back to F by pass 50 and was maintained in pass 100. Protein 3b had no amino acid changes. Protein 3c had four changes; V25G, F35L, Y42I, and N52H, and except for the F35L change, all other changes reverted back by pass 100.

The small envelope protein has one amino acid change (I71T) that was maintained in consecutive passages. The membrane protein had no amino acid changes. The nucloecapsid protein had one change L18R between passage 50 and 100, and protein 7a had one amino acid change L39M also between passage 50 and 100. The 7b protein, which starts at nucleotide position 28,462–29,082 and is 206 amino acids in length, was found to be truncated at passage 50 resulting in a 48 amino acid protein, which was also observed in passage 100 (Table 2).

Comparison with other group I CoV

Phylogenetic analysis (Fig. 1) for the full genome of the virus strains listed in Table 3 showed that FCoV/FIPV79-1146/USA passage 1, 8, 50, and 100 (accession number DQ010921) viruses group closely together (~99.8 or 99.9% similarity). The live attenuated FCoV/DF-2/USA vaccine virus (accession number DQ286389) also fell into the same clade (~99.2% similarity) and all are Type II viruses. The other FIPV viruses included in the analysis were, FCoV/FECV/Black/1970 strain (accession number EU186072), which had ~88% similarity, FCoV/FIPV/C1JE (accession number DQ848678.1), which had 83.6% similarity, and FCoV/INTU156p/2007 (accession number GQ152141), which had 92.2% similarity, when aligned to the viruses sequenced in this study. The Canine CoV/NTU336/F/2008 and the TGEV/Purdue/Pur46-Med and TGEV/Purdue/P126 are also grouped with Type II FCoV.

Simplot analysis of the full-length genome of FCoV/FIPV/FCoVWSU79-1146 pass 1 with selected FCoV,
CCoV, TGEV, and PRCV viruses showed that the polyprotein 1a, which comprises the first 10,000 nucleotides of the genome was found to be feline like having 82% sequence similarity or greater as seen in (Fig. 2 Box I.). Simplot analysis also showed that group II FCoVs are more closely related to TGEV, CCoV, and PRCV in the region of the viral polymerase than to Type I FCoV (Fig. 2 Box II). The FCoV type II viruses have low sequence similarity in the S protein to CCoV, TGEV, and PRCV (∼10% or less) (Fig. 2 Box III). The 3′ end of the genome comprising gene N and 7ab appears to be most similar to FCoV (Fig. 2 Box IV).

**Mutation rates**

The mutation rate was calculated for the full-length genomes of FCoV/FIPV/FCoVWSU79-1146 pass 1 to 100 in tissue culture and found using a linear regression analysis, to have a mutation rate of $6.3 \times 10^{-6}$ substitutions/site/tissue passage level. The evolutionary rate, calculated by BEAST, was estimated to be $5.08 \times 10^{-6}$ substitutions/site/tissue passage level.

**Growth curve analysis**

The growth curve analysis of FCoV/FIPV/FCoVWSU97-1146 passage 2, 9, and 51 show a slightly slower growth but overall higher titer as the virus is passed (Fig. 3). The passage 2 virus reached a peak titer of $1 \times 10^6$ PFUs at 24-h post-inoculation, whereas the peak titer of passage 9 was $1 \times 10^7$ PFUs at 48-h post-inoculation and passage 51 had a peak titer of $1 \times 10^8$ PFUs at 60-h post-inoculation.

**Discussion**

In this study, we identified the genetic differences in FCoV, FIPV WSU79-1146, as the virus was passaged in A-72 canine cells [12]. The etiology of clinical FIP in domestic...
cats is unclear, but it is thought to occur either by mutation(s) in an endogenous FECV, by infection with an exogenous FIPV, or both. To help elucidate the origin of FIPV, we examined the genetic differences, mutation rate, and growth kinetics of FCoV at different passage levels in cell culture to provide information regarding the capacity of this virus to change and adapt in a new host.

Sequence analysis of FIPV WSU 79-1146 at passage 1, 8, and 50 showed very few nucleotide changes or no changes in many of the genes. The Nsp 2 protein had 3 changes, which did not revert in subsequent passages making it the most variable protein. The Nsp 2 protein contains hydrophobic residues that are predicted to anchor the replication complex of the virus to the Golgi and it was

**Table 2** Location of open reading frames (all numbers correspond to the ORF’s individually), size of protein, and differences that occurred in passages of the virus in *Feline Coronavirus* 79-1146, pass 8 and pass 50

| ORF nucleotide position | Translation product (amino acids) | Amino acid identity differences WSU 791146 |
|-------------------------|-----------------------------------|------------------------------------------|
|                         |                                   | Pass O | Pass B | Pass 50 | Pass 100a                  |
| ORF 1a 311-12439        | Poly proteins la (4042)           | See Table 1 |
| ORF lab 311-20439       | Poly protein lab (6653)            | See Table 1 |
| ORF S 20436-24794       | Spike glycoprotein (1452)          | 599-T  | 599-A  | 599-T  | 599-T                      |
|                         |                                   | 743-A  | 743-A  | 743-A  | 743-V                      |
|                         |                                   | 1325R  | 1325R  | 1325R  | 1325E                      |
| ORF 3a 24SG1-2507G      | Accessory protein 3a (71)         | 61-F   | 61-S   | 61-F   | 61-F                       |
| ORF 3b 25021-25236      | Accessory protein 3b (71)         | –      | –      | –      | –                           |
| ORF 3c 25470-25967      | Accessory protein 3c (165)        | 25-V   | 25-G   | 25-V   | 25-Vb                      |
|                         |                                   | 35-F   | 35-F   | 35-L   | 35-Lb                      |
|                         |                                   | 42-Y   | 42-I   | 42-Y   | 42-Yb                      |
|                         |                                   | 52-N   | 52-H   | 52-N   | 52-Nb                      |
|                         |                                   | 15 AA rearrangement |
| ORF E 25354-26202       | Small membrane prole hi (82)      | 71-I   | 71-T   | 71-T   | 71-T                       |
| ORF M 26213-27001       | Membrane protein (262)             | –      | –      | –      | –                           |
| ORF N 27014-28147       | Xuckocapsid protein (377)         | 18-L   | 18-L   | 18-L   | 18-R                       |
| ORF 7a 25131-28457      | Accessory protein 7a (108)        | 39-L   | 39-L   | 39-L   | 39-M                       |
| ORF 7b 25462-29082      | Accessory protein 7b (206)        | –      | –      | trun @ 48A.A trun. @ 48A.A |

*a* FIPV WSU 79-1146 p100 was sequenced by Dye and Siddell [5]

*b* Indicates that amino acid position is not consistent with previous passages due to a rearrangement in pass100

**Fig. 1** The phylogenetic relationship among different Coronavirus isolates as inferred from Neighbor-Joining method in MEGA 5. Data for complete genome was subjected to 1,000 bootstrap replicates to produce a majority rule consensus tree. Highlighted in yellow are all FCoV that have been reported to cause FIPV (Color figure online)
found to block phosphorylation of dIF-2alpha shutting down host protein synthesis [13]. Changes in Nsp 2 could affect the pathogenicity of the virus.

The accessory protein 3c had more nucleotide changes (four changes) than any of the other proteins but only one change F35L was maintained in subsequent passages. Protein 3c was also truncated to a 165 amino acid protein in passages 1, 8, 50, and 100 compared to the previously reported 238 amino acid 3c protein in FECV [14]. The FCoV accessory protein 3c is a membrane-spanning protein that is predicted to have similar topology to SARS-CoV 3A [15]. The SARS-CoV 3A protein is the largest of all accessory proteins containing 274 amino acids. This protein localizes to the Golgi, the plasma membrane and intracellular vesicles [16]. One study investigated the function of 3A and found that it contributes to the cytotoxicity of SARS-CoV and causes intracellular vesicle formation, which is thought to be necessary for Golgi fragmentation during virus infection [17]. However, the depletion of SARS-CoV 3A, in a previous study, did not reduce the levels of RNA isolated from infected cells or media indicating this protein and double membrane vesicles may not be necessary for replication [17]. Another possibility is that this protein functions as a means to rearrange the Golgi for nonlytic release of virus particles. Such is the case with Poliovirus, which has been identified as using intracellular vesicles and double membrane vesicles for nonlytic release of virus particles [18].

The majority of differences noted in the 3c protein as the virus was passed in cell culture could also be interrupted as this protein not playing an important role in viral replication. In a previous study, the entire 3abc group specific gene cluster was deleted from FIPV 79-1146 virus resulting in no significant difference in viral growth in cell culture, but the virus was shown to be attenuated and immunogenic in cats [19]. A recent study showed that intact 3c genes appear to be essential for replication in the intestines and while some FIPV isolates have intact 3c genes the majority of FIPV isolates contained more nonsynonymous nucleotide changes in the 3′ one-third of the 3c protein than FECVs [10]. It is interesting to note that ORF 3abc and spike have been identified in a genome comparison between Ferret enteric coronavirus (FEC) and the Ferret Systemic Coronavirus to have the majority of differences [20]. Both of these viruses cause similar diseases to FECV and FIPV respectively in ferrets [20].

### Table 3 List of Coronavirus isolates and strains included in the sequence and phylogenetic analysis

| No. | Isolate/strain       | Accession no. | Origin         |
|-----|----------------------|---------------|----------------|
| 1   | FCoV/UE11/Netherlands/2007 | FJ938052.1    | Netherlands    |
| 2   | FCoV/UE10/Netherlands/2007 | FJ938059.1    | Netherlands    |
| 3   | FCoV/UE9/Netherlands/2007  | FJ938062.1    | Netherlands    |
| 4   | FCoV/UE54/Netherlands/2010 | JN183883.1    | Netherlands    |
| 5   | FCoV/UE30/Netherlands/2008 | HQ392472.1    | Netherlands    |
| 6   | FCoV/UE2/California/1993  | FJ938060.1    | California     |
| 7   | FCoV/FECV/Black/1970     | EU186072.1    | USA            |
| 8   | FCoV/UE47/Netherlands/2010 | JN183882      | Netherlands    |
| 9   | FCoV/UE16/Netherlands/2007 | FJ938058.1    | Netherlands    |
| 10  | FCoV/FIPV/1/2003        | DQ848678.1    | –              |
| 11  | FCoV/FIPV WSU79-1146/p8 | KC461236      | –              |
| 12  | FCoV/FIPVWSU79-1146/p50 | KC461237      | –              |
| 13  | FCoV/FIPVWSU791146/USA  | DQ010921      | USA            |
| 14  | FCoV/FIPV WSU79-1146/p1  | KC461235      | –              |
| 15  | FCoV/DFD-2/USA          | DQ286389.1    | USA            |
| 16  | FCoV/FECV/WSU79-1683    | –             | –              |
| 17  | TGEV/Purdue/Pur46-Mad   | NC_002306.2   | –              |
| 18  | TGEV/Purdue/P126        | DQ811788.1    | USA            |
| 19  | CCuV/NTU36/F/2008       | GQ477367      | Taiwan         |
| 20  | FCoV/NTU156/p/2007      | GQ152141      | Taiwan         |
| 21  | WBCoV/US/OH-WD358-TC/  | FJ425184.1    | USA            |
|     | 1994                  |               |                |
| 22  | SDCoV/US/OH-WD-388-TC/  | FJ425188.1    | USA            |
|     | 1994                  |               |                |
| 23  | HCoV/4408/Germany/1988 | FJ415324      | Germany        |
| 24  | SaCoV/US/OH1/2003      | EF424621.1    | USA            |
| 25  | GCoV/US/OH3-TC/2006    | EF424622.1    | USA            |
| 26  | CGCoV/US/OH3/2006     | EF424624.1    | USA            |
| 27  | EqCoV/NC99            | EF446615.1    | –              |
| 28  | BatCoV/AFCd62/HongKong | NC_010437.1   | China          |
| 29  | TGEV/SC-V/China       | DQ443743      | China          |
| 30  | TGEV/TGEVMiller16/16   | DQ811785.1    | –              |
| 31  | PRCV/PRCVIsu-1/18     | DQ811787.1    | –              |

Origin included if available
reflecting the ability of the higher passaged virus to modulate apoptosis.

Mutation rate is a crucial parameter driving viral evolution. The ability to undergo a high rate of genetic change facilitates the development of diverse viral subpopulations that can emerge as new variants capable of cell tropism and pathogenicity shifts. It is still unclear if FIPV arises from point mutations that occur during the FECV replication or if they are two closely related circulating viruses or if both play a role in development of FIP in cats. It must be noted that FCoV has been shown to exist in quasispecies [23]. It is interesting to note that the synonymous mutation rate in CoV’s is $1.2 \times 10^{-3}$ [24]. However, in this study, the mutation rate was calculated to be $5.08-6.3 \times 10^{-6}$. The reason for the low mutation rate is unclear could be due to the different selection pressures that occur in vitro and in vivo. In this study, we showed that FIPV has the capacity for change specifically in nsp 2, 3c, and 7b as it adapts to cell culture, highlighting the importance of continued studies to elucidate changes in FECV that could potentially lead to FIP in cats.

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