Comparative sequence analysis of the distal one-third of the genomes of a systemic and an enteric ferret coronavirus

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ABSTRACT

Ferret systemic coronavirus (FRSCV) infection is associated with an emerging, highly fatal disease of ferrets. Enhanced macrophage tropism and the resulting induction of pyogranulomatous lesions are shared with feline infectious peritonitis virus (FIPV) infection in cats, but are not features of ferret enteric coronavirus (FRECV) infection. Comparative sequence analysis of the distal one-third of the genomes of one FRSCV and one FRECV strain showed that these two ferret coronaviruses share >96% nucleotide sequence identities in the membrane (M), nucleocapsid (N) and non-structural protein genes (partial polymerase, open reading frames [ORFs] 3 and 7b). The envelope (E) protein gene showed a moderate nucleotide sequence similarity of 91.6%. In contrast, nucleotide and amino acid sequence similarities observed with the spike (S) protein were only 79.5 and 79.6%, respectively. Twenty-one amino acid differences within a 195–199-amino acid C-terminal portion of the S protein were conserved between 3 strains each of FRSCV and FRECV. Both systemic and enteric strains were found to carry a single ORF 3 gene with truncated proteins observed in two out of three FRSCV strains examined. The two enteric strains analyzed each contained an intact ORF 3 gene. Phylogenetically, FRSCV is more closely related to FRECV than to other group 1 coronaviruses.

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1. Introduction

An emerging systemic disease in ferrets characterized by feline infectious peritonitis (FIP)-like clinical signs and lesions, has been recently observed in ferrets across the U.S. and also in Europe (Garner et al., 2008; Juan-Sallés et al., 2006; Martinez et al., 2006, 2008). Clinical findings commonly observed in the FIP-like disease in ferrets include anorexia, weight loss, diarrhea and the presence of large palpable intra-abdominal masses. Strikingly similar to FIP is the presence of widespread granulomas on serosal surfaces and within the parenchyma of the abdominal and thoracic organs. Histopathologic examination shows a systemic pyogranulomatous inflammation involving the mesenteric adipose tissue, lymph nodes, liver, lungs, kidney, spleen, pancreas and adrenal glands. Moreover, the immunohistochemical detection of coronavirus antigen in the cytoplasm of intra-lesional macrophages is consistent with what has been observed in tissues of cats with FIP.

FIP is a fatal, multi-systemic, immune-mediated disease of cats caused by FIP viruses (FIPV), postulated to be virulent mutants arising spontaneously from persisting low pathogenic or non-pathogenic feline enteric coronavirus strains (De Groot-Mijnes et al., 2005; Poland et al., 1996; Vennema et al., 1998; Herrewegh et al., 1995; Rottier et al., 2005; Chang et al., 2009; Pedersen, 2009). More recently, a study by Brown et al. (2009) called into question this in vivo mutation hypothesis of FIP pathogenesis. Their findings supported another hypothesis, namely, that genetically distinctive avirulent and virulent forms of feline coronavirus (FCoV) co-circulate in natural populations.

In a recent publication (Garner et al., 2008), we reported the detection of coronaviral RNA in the tissues of the affected ferrets by amplification of a portion of the coronavirus spike gene using consensus RT-PCR. A unique 599-bp partial spike sequence of the ferret systemic coronavirus (FRSCV) was derived and analyzed. This sequence was found to be more similar (77% deduced amino acid sequence similarity) to the previously described (Wise et al., 2006) ferret enteric coronavirus (FRECV) than to other group 1 coronaviruses, feline coronavirus, transmissible gastroenteritis virus (TGEV) of swine and canine coronavirus (CCV) (71–73% deduced

amino acid sequence similarities). FRECV is associated with a different disease in ferrets, epizootic catarrhal enteritis (ECE) (Williams et al., 2000). ECE is a highly contagious diarrheal disease of ferrets associated with a mortality rate of <5%. When first recognized, the disease was more severe in older ferrets but often mild and subclinical in juveniles. We previously characterized FRECV, and found it to be a novel group 1 coronavirus (Wise et al., 2006), based upon analyses of partial sequences of the polymerase (pol), spike (S) and membrane (M) protein genes and the complete sequence of the nucleocapsid (N) gene. This study is the first report on the genetic relationship between one systemic (FRSCV MSU-1) and one enteric (FRECV MSU-2) ferret coronavirus strain, based upon a comparative analysis of their 3′-most 8.3–8.6 kb genomic sequences.

2. Materials and methods

2.1. Samples and strain designations

Tissue samples were obtained from a ferret that had exhibited clinical signs and lesions of a FIP-like disease. Immunohistochemical staining of the affected tissues (lymph nodes, lung, liver, spleen, and brain) with anti-feline coronavirus antibody was positive. Total RNA was extracted from the tissue specimens using the QIAGEN RNeasy Mini Kit (Valencia, CA). The systemic ferret coronavirus analyzed in this study is hereafter referred to as strain FRSCV MSU-1.

Fecal samples from a ferret diagnosed with ECE were obtained. RNA was extracted and was found positive for FRECV by an N-gene specific RT-PCR (Wise et al., 2006). The enteric ferret coronavirus strain was designated FRECV MSU-2.

2.2. Deriving the sequences of the distal one-third of the genomes of FRSCV MSU-1 and FRECV MSU-2

The primer sequences pertaining to these experiments are presented in Supplementary Material Table 1. Initially, partial S, M and N sequences were obtained for each ferret coronavirus strain using coronavirus consensus RT-PCR primers (S primers 55 and 56, and M-N primers 25 and 24 (Tobler and Ackermann, 1996), as previously described (Wise et al., 2006) The partial S sequence (approximately 600 bases) is located less than 200 nucleotides from the 3′ end of the gene, while the M-N target consists of approximately the last 350 nucleotides of the M gene extending into approximately the first 350 nucleotides of the N gene.

To bridge the approximately 2 kb gap between the 3′ end of the S gene and the 3′ end of the M gene, primers were designed from partial sequences of the S and M genes. The QIAGEN OneStep RT-PCR Kit was used with a 0.5 μM final primer concentration in a 50 μl reaction volume. Cycling conditions were as follows: RT step at 48 C for 30 min, hotstart denaturation at 95 C for 15 min, followed by 40 cycles of 94 C for 30 s, 55 C for 30 s and 72 for 2 min. Final extension was at 72 C for 7 min.

The rest of the N gene plus downstream sequence up to the 3′ untranslated region (UTR) of the genome was obtained by performing a 3′ RACE, as previously described (Wise et al., 2006). Gene-specific forward primers FRECV N-GSP and FRSCV N-GSP were designed from available partial 5′ end sequence of the N genes.

For FRSCV MSU-1, an additional 3′ RACE product that starts from the 3′ end of the S gene, was obtained using FRSCV S-GSP as the forward primer. Complementary DNA was synthesized according to the manufacturer's protocol (3′ RACE System, Invitrogen, Carlsbad, CA). PCR of the target cDNA was performed using the Accuprime Taq DNA Polymerase High Fidelity Kit (Invitrogen). The 50 μl reaction mix contained 2 μl of the cDNA template, 0.2 μM of S-GSP and AUAP primers, 5 μl of 10× Buffer II, 1 μl of Taq and proofreading enzymes, and sterile water. Cycling conditions were as follows: hot-

start denaturation at 94 C for 2 min, followed by 35 cycles of 94 C for 15 s, 55 C for 30 s, and 68 C for 6 min. Final extension was at 68 C for 7 min.

To derive the remaining S gene sequence (~3.2 kb) upstream of the already sequenced 3′ end of the gene, 3 overlapping PCR fragments (S Codehop 1, S Codehop 2 and Pol 1b-S Codehop 3) were amplified sequentially by designing degenerate forward primers via the CODEHOP (consensus-degenerate hybrid oligonucleotide primer) strategy (Rose et al., 2003) and specific reverse primers from already determined sequence data. The CODEHOP degenerate PCR primers were obtained by identification of conserved blocks of amino acids between the S and pol1b protein sequences of the group 1 animal coronaviruses FIPV, CCV and TGEV. The QIAGEN OneStep RT-PCR was used for amplifying each of the 3 fragments, using 0.5 μM final primer concentrations in 50 μl reaction volumes. It was necessary to add the Q solution for S Codehop 2 amplification. RT and pre-denaturation steps of 50 C for 30 min and 95 C for 15 min were carried out for all 3 fragments. This was followed by 10 cycles of touchdown PCR with gradually decreased annealing from 60 C to 55 C (for S Codehop 1), from 63 C to 56 C (for S Codehop 2), and from 60 C to 51 C (for Replicase-S Codehop 3), followed by an additional of 35 cycles of 94 C for 30 s, the respective annealing temperatures after touchdown for 30 s and extension at 72 C for 2–3 min. Final extension was at 72 C for 7 min.

The PCR products were extracted from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN). Purified PCR products were submitted to the Research Technology Support Facility of Michigan State University for automated sequencing with both forward and reverse amplification primers. This was followed by primer-walking with newly designed 5′ and 3′ primers derived from acquired sequence data. All specific primers were designed using the OLIGO 6 primer analysis software (Molecular Biology Insights, Cascade, CO).

Verification of the first set of sequence data was done by direct sequencing of 6 overlapping fragments (~1.5–2 kb each) using newly designed sequence-specific primers (Supplementary Material Table 2). The final sequence data have an average coverage of 4x.

2.3. Sequencing of the partial spike genes of additional FRSCV and FRECV strains

Samples from additional ECE and FIP-like case submissions were used to identify and analyze additional strains. Consensus RT-PCR for the S gene was used to derive the partial gene sequences of 2 additional FRSCV strains (FRSCV MSU-S and FRSCV WADL) and 1 FRECV strain (FRECV 1202). FRSCV WADL was the same strain from which the S gene was partially sequenced in a previous study (Garner et al., 2008). The partial S sequences obtained from these additional strains were compared to the corresponding sequences of FRSCV MSU-1 and FRECV MSU-2 (this study) and FRECV MSU-1 (previously designated as FECV MSU1; GenBank Accession DQ340561; Wise et al., 2006).

2.4. Sequencing of the ORF 3 regions of additional FRSCV and FRECV strains

ORF 3 region sequences were also obtained from strains FRECV 1202, FRSCV MSU-S and FRSCV WADL, and compared to the sequences of FRECV MSU-2 and FRSCV MSU-1. The ORF 3 region was amplified using the S-E (spike-envelope) region primer set, targeting a 0.8–1 kb region (Supplementary Material Table 1). Amplification was performed following the same protocol used for the S-M region RT-PCR, except for an annealing temperature of 52 C and an extension time of 1 min and 15 s.

2.5. Sequence and phylogenetic analyses

BLAST (Altschul et al., 1990) similarity searches against the GenBank database were performed to determine the nature of the sequence obtained. Sequence assembly and analyses, including open reading frame (ORF) searches and multiple alignments of nucleotide and predicted amino acid sequences using the Clustal W (Thompson et al., 1994), were done with the Lasergene biocomputing software (DNASTAR, Inc., Madison, WI). Phylogenetic trees were constructed with the Treecon software package (Van de Peer and De Wachter, 1994), using the neighbor-joining (Saitou and Nei, 1987) and 100 bootstrap analyses (Efron and Gong, 1983; Felsenstein, 1985). Sequence alignment data generated with Lasergene were converted to the Treecon format using the ForCon software (Raes and Van de Peer, 1999).

2.6. Sequences and GenBank accession numbers

The following are the GenBank accession numbers of the sequences used in the construction of the phylogenetic trees: DQ010921 (FIP 79–1146); EU186072 (FCoV Black); D13096 (CCV INSAVC); DQ112226 (CCV CB/05); DQ340562 (FRECV MSU-1); EF192159, EF192155 and EF192157 (RDCoV pol 1b, S and N, respectively); EF192160, EF192156, and EF192158 (CFBCoV pol 1b, S and N, respectively); DQ811786 (TGEV); DQ811787 (PRCV); NC_003436 (PEDV); NC_002645 (HCoV 229E); EU371564 (SARS); GU338456 (FRSCV MSU-1, this study); GU338457 (FRECV MSU-2, this study).

The partial S and complete ORF 3 gene sequences obtained from additional FRSCV and FRECV strains were deposited in GenBank under accession numbers GU459055–GU459060.

2.7. Development of two genotype-specific RT-PCR assays for the detection of ferret coronaviruses in clinical samples

A multiple alignment of the partial S nucleotide sequences (approximately 600 bases) of FRSCV and FRECV strains, corresponding to the amino acid alignment in Fig. 1, was performed (data not shown). The sequences were highly conserved among strains within a pathotype group. The 3 FRSCV strains shared an average similarity of 94.7%, while the 3 FRECV strains had a 91.8% average identity. Average similarity between enteric and systemic strains was only 78.5%. These observations made it possible to design two genotype-specific RT-PCR primer sets. Fig. 2 shows the aligned PCR target regions (sense strand) within the S genes of FRSCV MSU-1 and FRECV MSU-2. The positions of the genotype-specific primers for each virus are underlined. The genotype 1-specific primers, forward, 5′-CTGGTGTTTGTGCAACATCTAC-3′, and reverse, 5′-TCTATTTGCACAAAATCAGACA-3′, are based upon the FRSCV sequences, and amplify a 157-bp product. The genotype 2-specific primers, forward, 5′-GGCATTTGTTTTGATAACGTTG-3′, and reverse, 5′-CTATTAATTCGCACGAAATCTGC-3′, are based upon the FRECV sequences, and amplify a 146-bp product. These primer sets were used at a final concentration of 0.5 μM in a 50 μl reaction volume, using the QIAGEN One-Step RT-PCR Kit. The RT-PCR assays were run with the following cycling parameters: an RT step of 50 C for 30 min, pre-denaturation at 95 C for 15 min; followed by 40 cycles of 94 C for 30 s, 53 C for 30 s and 72 C for 30 s. Final extension was at 72 C for 7 min. The products were analyzed in 2% pre-stained agarose gels and visualized by UV transillumination.

3. Results

3.1. Characterization of the sequences spanning the distal one-third of the genomes of FRSCV MSU-1 and FRECV MSU-2

Sequences of the distal third of the genomes of systemic ferret coronavirus, FRSCV MSU-1 (8.3 kb), and enteric ferret coronavirus, FRECV MSU-2 (8.6 kb), were derived. Each sequence spans the last 317 nucleotides of the polymerase 1b gene, the full coding sequences of structural protein genes (S, E, M and N) and the non-structural protein (nsp) genes 3b or 3c-like (ORF 3), 3x-like and 7b (ORF7). Partial sequence was also derived from the 3′ untranslated

Fig. 1. Alignment of the deduced partial amino acid sequences of the spike proteins of FRSCV MSU-1, FRSCV MSU-S, FRSCV WADL, FRECV MSU-2, FRECV-MSU1 and FRECV 1202. Residues that match the majority sequence are shown as dots.

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FRSCV MSU-1    5'- ctggtgtttgtgcaacatctactaataataaaactatgggtttttattgttaaagatgtgtcgttgacgttgtttaaaaatcac        80
FRECV MSU-2    5'- ....ca.....tt ------------.g.t.acg.t.....g.....c..g.....t..................c...t              68


FRSCV MSU-1    gatggcaggttctacttgacaccgcgtacaatgtacgaaccgcgagttgcaactatgtctgattttgtgcaaatagaaag -3'        160
FRECV MSU-2    .... at.aa.............. a..... t.....t .. g........ c..g....gtg.a.....c....g...ta .t.. -3'         148
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Fig. 2. Alignment of the genotype-specific diagnostic PCR target regions within the spike genes of FRSCV MSU-1 and FRECV MSU-2. Matching nucleotides are shown as dots. The broken line indicates a deletion. Primer sequences are underlined.

region (UTR) of the genome. The genomic organization observed for each virus was consistent with that of a typical coronavirus. Each intact gene was preceded, a few nucleotides upstream its 5′ end, by the recognized consensus transcription-regulating sequence (TRS), 5′-CTAAAC-3′, of coronaviruses (Budzilowicz et al., 1985; Horsburgh et al., 1992; Kapke and Brian, 1986; Snijder et al., 2003; Spaan et al., 1988). In both viruses, the TRS for the E and 7b genes each exhibited a one-base mismatch at the 5′ end, appearing as 5′-GTAAAC-3′ for the former, and 5′-TTAAAC-3′ for the latter. The highly conserved 10-base sequence, 5′-GGGAAGAGCT-3′, found in the 3′ UTR of all coronaviruses, was also noted, both in FRSCV and FRECV. Reminiscent of the observation made for FRECV MSU-1 (Wise et al., 2006), the 10-base sequence was found 170 bases downstream ORF 7b and had the same 2 base mismatches, a T instead of a G in the first base, and a G instead of an A at the seventh base.

Each ORF was analyzed by BLAST against the GenBank database and was confirmed to be of coronavirus origin. Table 1 shows the summary of the ORFs identified for each virus strain, the gene and predicted protein sequence lengths, and the nucleotide and amino acid sequence similarities between FRSCV MSU-1 and FRECV MSU-2. Among the nsp genes identified, a single open reading frame, ORF 3, between the S and E genes was found for both viruses. BLAST analysis of the putative ORF 3 protein showed highest significant similarities (56–59%) to nsp 3c of CCV and FCoV, and to the 3c homologue, nsp 3b of TGEV. Two more putative nsp genes observed were the two coronavirus ORF 7-like genes downstream of the N gene, namely, 3x-like and 7b, as previously observed in the FRECV MSU-1 strain (Wise et al., 2006). However, FRSCV MSU-1 only contained a 45-bp remnant of the 3x-like ORF because of a 113-bp deletion, 33 bases downstream of the start codon, resulting to a frameshift that prematurely ends the ORF. FRECV MSU-2 shared very high sequence similarities to previously reported partial S and M, full N, 3x-like and 7b protein sequences of FRECV MSU-1 (96.4–100%). FRSCV MSU-1 shared 94.6–100% similarities with FRECV MSU-1 for the M, N and 7b sequences, but exhibited a marked sequence difference (only 77.4% identity) for the partial S protein sequence.

The ORF lengths were identical for the pol, E, M, N and 7b genes of FRSCV MSU-1 and FRECV MSU-2. The 4.35 kb S sequence of FRECV MSU-2 is 24 nucleotides shorter, observed as a deletion of 8 amino acid residues in a pairwise comparison of predicted protein sequences (Fig. 3). ORF 3 of FRSCV MSU-1 is only 252 bases (83 amino acids) long compared to 744 bases (247 amino acids) in FRECV MSU-2. In the ORF 3 sequence alignment in Fig. 4, it can be observed that the ATG start codon at position 9, found in the enteric strain, has been replaced with a GTG in the systemic strain, resulting in a delayed start site further downstream at nucleotide position 311 for FRSCV MSU-1. In addition, the systemic strain has a 193-base deletion that corresponds to positions 228–420 within the intact ORF 3 gene of FRECV MSU-2.

Pairwise alignment of the derived partial genomic sequences of FRSCV MSU-1 (8.3 kb) and FRECV MSU-2 (8.6 kb) showed only a similarity of 87%. For the individual ORFs, the genes that exhibited the highest nucleotide sequence similarities (96.2–98.1%) between the two viruses were the partial pol1b, full M, N and 7b genes. Sim-

ilarities based on the deduced amino acid sequences for these 4 genes ranged from 94.6 to 98.1%. Similarity of the truncated putative ORF 3 nsp of FRSCV MSU-1 to the corresponding sequence in FRECV MSU-2 was 96.3%. The E gene exhibited intermediate nucleotide (91.6%) and amino acid (87.8%) similarities. However, significant divergence was observed in the S gene, with nucleotide and amino acid sequence similarities of only 79.5 and 79.6%, respectively. The alignment of the deduced S protein sequences of FRSCV MSU-1 and FRECV MSU-2 (Fig. 3) shows the amino acid substitutions to be randomly distributed throughout with up to 12 consecutive substitutions noted in the middle third of the sequence.

3.2. Analysis of partial spike gene sequences of additional systemic and enteric ferret coronaviruses

To determine whether the marked difference (79.6% amino acid similarity) observed in the S genes of FRSCV MSU-1 and FRECV MSU-2 may possibly be linked to pathotype difference, and not to random heterogeneity of the S sequence in ferret coronaviruses, a limited number of additional strains from different geographic locations were analyzed. The partial S sequences of two other systemic strains, FRSCV MSU-S and FRSCV WADL, and one other enteric strain, FRECV 1202, were determined by consensus S gene RT-PCR. Their deduced amino acid sequences were aligned with corresponding sequences of FRSCV MSU-1, FRECV MSU-2 and FRECV MSU-1 (Fig. 1). The partial sequence being analyzed corresponds to residues 1195–1393 of the FRSCV MSU-1 S protein in Fig. 3. The alignment in Fig. 1 shows that 21 amino acid substitutions observed between the systemic and enteric viruses were conserved within a virus pathotype. Moreover, the same four residue deletion was consistently observed in each of the three FRECV strains examined in comparison to the three strains of FRSCV. The systemic strains shared an average identity of 94.3% with each other. The average similarity shared between two enteric strains was 92.3%. Average similarity of the partial spike sequences between the two pathotypes was only 80.1%.

3.3. Phylogenetic analyses

The deduced amino acid sequences of the partial polymerase, full S, E, M and N proteins of FRSCV MSU-1 and FRECV MSU-2 were aligned with corresponding sequences of previously reported group 1 coronaviruses. The phylogenetic trees derived are shown in Fig. 5. Sequence similarities between ferret coronavirus and representative group 1 coronaviruses ranged from 66.3 to 74% for the partial pol, 43 to 63.6% for S, 32.5 to 64.2% for E, 47.3 to 66.7% for M, and 34.6–60.2% for N. The two ferret viruses were observed to segregate into their own distinct cluster within the group.

3.4. Analysis of ORF 3 region sequences of systemic and enteric ferret coronaviruses

ORF 3 region sequences of additional strains, FRECV 1202, FRSCV WADL, and FRSCV MSU-S, were determined and compared to the sequences of FRECV MSU-2 and FRSCV MSU-1. Enteric strain FRECV

Table 1
Comparison of FRSCV MSU-1 and FRECV MSU-2 sequences spanning the distal one-third of the genome.

Gene/protein name	ORF length in bp		No of amino acids		% Amino acid similarity between FRSCV and FRECV	% Nucleotide similarity between FRSCV and FRECV
	FRSCV	FRECV	FRSCV	FRECV		
Pol 1b[a]	317	317	104	104	98.1	98.1
S	4374	4350	1457	1449	79.6	79.5
ORF 3	252	744	83	247	96.3[b]	96.4[b]
E	249	249	82	82	87.8	91.6
M	792	792	263	263	97	97.7
N	1125	1125	374	374	94.9	96.2
3x-like	45	225	14	74	nd	nd
7b	615	615	204	204	94.6	96.6

nd = not done.
[a] Partial sequence only.
[b] FRSCV sequence compared to corresponding partial sequence of FRECV.

1202 and systemic strain FRSCV MSU-S both have an intact ORF 3, consisting of 744 nucleotides, similar to that of FRECV MSU-2. Similarity of the intact putative ORF 3 protein between the two enteric strains was only 79.8%. The ORF 3 protein of FRSCV MSU-S was only 78.1% similar to FRECV 1202, but shared a much higher identity with enteric strain, FRECV MSU-2, at 93.9%. The ORF 3 sequence of systemic strain FRSCV WADL had a deletion of 86 bases (positions 256–341 of FRECV MSU-2 in Fig. 4) that overlaps the 193-base deletion in FRSCV MSU-1. This deletion results to the splitting of ORF 3 into two shorter ORFs in FRSCV WADL—a proximal ORF, expressing a putative protein of 98 amino acids with a start site similar to that of FRECV MSU-2, and a distal ORF that translates into a

Fig. 3. Alignment of the deduced amino acid sequences of the spike proteins of FRSCV MSU-1 and FRECV MSU-2. Conserved residues are shown as dots.
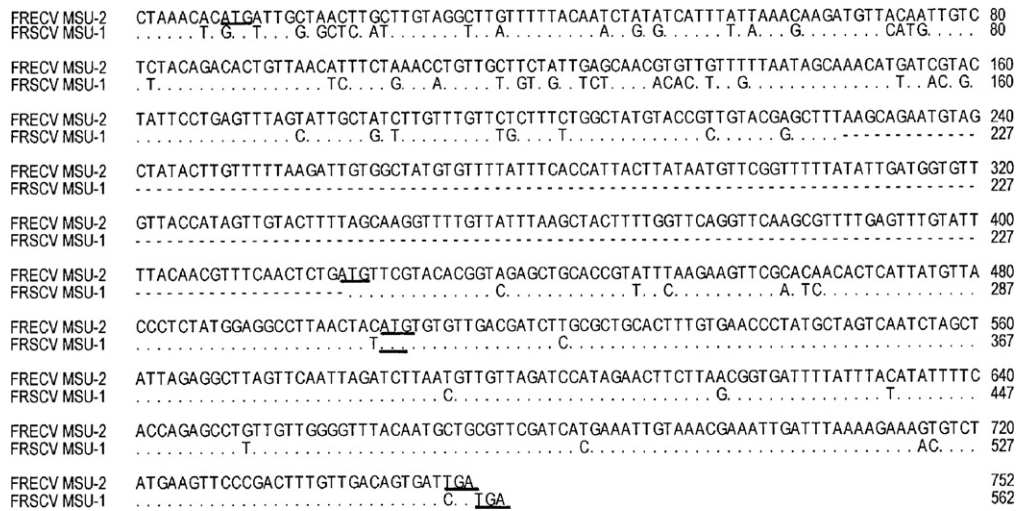
Fig. 4. Alignment of the ORF 3 region sequences of FRECV MSU-2 and FRSCV MSU-1. Matching nucleotides are shown as dots. Deletions are represented by broken lines. ATG and TAG or TAA codons are underlined.

110 amino acid protein, whose ATG corresponds to position 420 in FRECV MSU-2 (Fig. 4). ORF 3 region sequences of systemic strains FRSCV WADL and MSU-S were highly similar at 95.5%. The corresponding ORF 3 region sequence of FRSCV MSU-1 appeared more distant with an average identity of only 85.75% to either systemic strain. These results indicate high sequence variability in the ORF 3 region of the ferret coronavirus strains examined.

3.5. Detection of FRSCV or FRECV in specimens using the S-gene genotype-specific diagnostic RT-PCR primer sets

RNA from various tissue samples, from which strains FRSCV MSU-1, FRSCV-WADL and FRSCV MSU-S had been previously identified, were tested with the genotype 1-specific diagnostic primer set. The 157-bp S gene target was amplified from liver, lung, spleen,
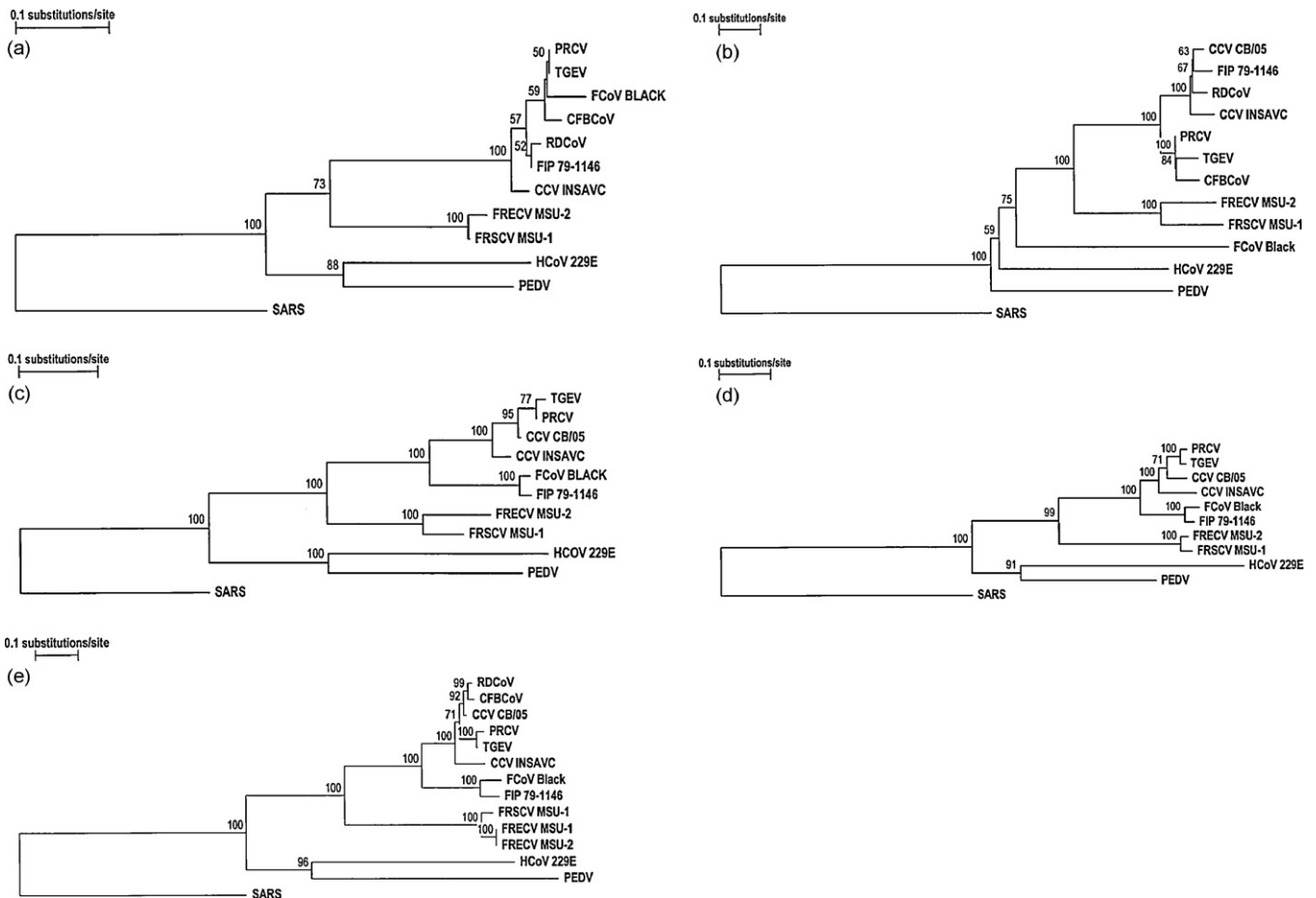
Fig. 5. Phylogenetic trees based on deduced partial amino acid sequences of the: (A) partial polymerase, (B) spike, (C) envelope, (D) membrane, and (E) nucleocapsid proteins of FRSCV MSU-1, FRECV MSU-2 and representative strains of group 1 coronaviruses. SARS virus was included as the outgroup sequence. Bootstrap values are indicated at the nodes.
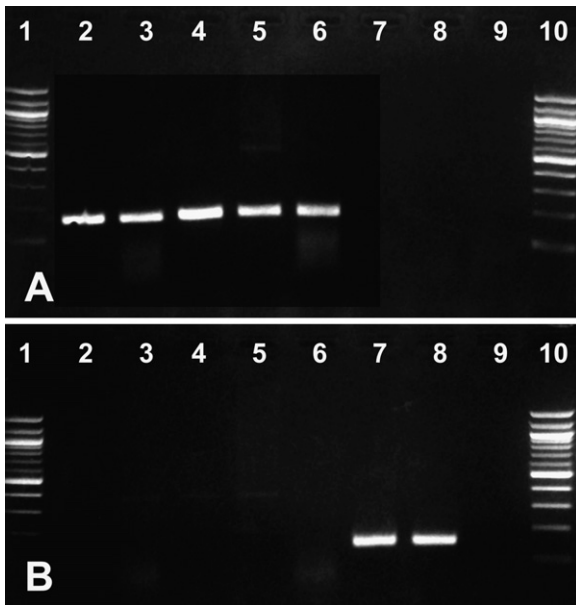
Fig. 6. S-gene genotype-specific diagnostic RT-PCR on clinical samples. (A) Genotype 1-specific assay, 157-bp positive amplicon observed only on Lanes 2–6. (B) Genotype 2-specific assay, 147-bp positive amplicon observed only on Lanes 7–8. Lane designations for both panels A and B: Lanes 1 and 10, 100-bp DNA ladder; Lane 2, FRSCV MSU-1 positive lung; Lane 3, FRSCV MSU-1 positive kidney; Lane 4, FRSCV WADL positive lymph node; Lane 5, FRSCV MSU-S positive spleen; Lane 6, FRSCV MSU-S positive intestine; Lane 7, FRECV MSU-2 positive feces; Lane 8, FRECV 1202 positive feces; Lane 9, negative control (sterile water).

kidney, brain, lymph node and intestinal tissues collected from ferrets affected with the FIP-like disease. Fig. 6A shows the specific 157-bp product amplified from each sample for a subset of these specimens. Enteric strains FRECV MSU-2 and FRECV 1202, did not amplify with the genotype 1-specific primers. Fig. 6B shows the results for the same set of samples tested in Fig. 6A, but using the genotype 2-specific primer set. The genotype 2-specific RT-PCR assay detected the 147-bp S gene target only in the samples known to contain FRECV strains.

4. Discussion

Ferret systemic coronavirus causes signs and lesions that are similar to those induced by FIPV in cats. The partial S protein sequence we initially derived from a FRSCV strain (Garner et al., 2008), was the first indicator that this virus appeared to be more similar to ferret enteric coronavirus than to any other group 1 coronavirus. The question then arose as to whether FRSCV strains could have evolved from FRECV by in vivo mutation, leading to the acquisition of a systemic virulent pathotype. The analogous situation in cats is enteric feline coronavirus infection, which causes a mild or asymptomatic localized enteric infection (Pedersen, 2009; Evermann et al., 1991; Rottier, 1999; Weiss and Navas-Martin, 2005; Addie et al., 2004) and the systemic highly fatal disease FIP, considered to be derived by spontaneous mutation during FCoV persistence (Evermann et al., 1991; Rottier, 1999; Weiss and Navas-Martin, 2005; Addie et al., 2004; De Groot-Mijnes et al., 2005; Poland et al., 1996; Vennema et al., 1998; Herrewegh et al., 1995; Rottier et al., 2005; Lai et al., 2007; Pedersen, 2009; Chang et al., 2009). The mutation from FCoV to FIPV is accompanied by a marked acquisition of macrophage tropism (Pedersen, 1987; Stoddart and Scott, 1989), which facilitates systemic spread (Rottier et al., 2005; Lai et al., 2007; Pedersen, 1987). The precise nature of this phenomenon is not entirely understood (Lai et al., 2007). In fact, enteric FCoVs and their counterpart FIPVs were observed to be genetically very similar (Poland et al., 1996; Herrewegh et al., 1995; Vennema et al., 1998), bearing only minor but crucial differences (Pedersen, 2009). Candidate genes speculated to play a role in the virulence shift include the S and group-specific genes 3abc, 7b and 7a (Haijema et al., 2004; Vennema et al., 1998; Kennedy et al., 2001). Using targeted RNA recombination, Rottier et al. (2005) have shown that the C-terminal 582-amino acid region of the FIPV 79–1146 S protein contains the amino acid changes that determine macrophage tropism. In this region of the S protein, a total of only 10 amino acid differences were noted between FIPV 79 and 1146 and its close relative, enteric strain FCoV 79–1683. A "D-to-A" substitution, upstream of the putative fusion peptide, was speculated to play a role in the transition of FCoV to FIPV. Pedersen (2009), in a comprehensive review of FIPV infection, states that mutation in the 3c gene is responsible for the FIP biotype. Similarly, Chang et al. (2009) analyzed the 3c gene in 27 feline enteric coronavirus (FECV)- and 28 FIPV-infected cats and found that the 3c gene was mutated in the majority (71.4%) of the cats with FIP.

An alternative to the in vivo mutation hypothesis of FIPV strain development was put forward by Brown et al. (2009) and was based on phylogenetic analyses of the M and nsp 7b gene sequences from FCoV-infected clinically healthy and sick cats. They observed that viral sequences from healthy cats were distinct from those of sick cats supporting the hypothesis that distinctive virulent and avirulent FCoV strains co-circulate in natural populations.

With the currently available data, it is still difficult to predict whether the systemic ferret coronavirus, FRSCV, is derived from its enteric counterpart, FRECV, by in vivo mutation, or is a co-circulating distinct strain. To resolve this, a significant number of additional strains from the same or different locations will have to be examined. It is also not clear at this point whether FRSCV originated as a result of a recombination between FRECV and another group 1 coronavirus, resulting in the acquisition of a very distinct S gene. A 3′–5′ scanning of the nucleotide sequence alignment for the two virus strains did not show an abrupt discontinuity in identity at the 3′ end of the S gene that would have been an indication of recombination. We noticed instead an increase in sequence divergence starting at the 200 bases adjacent to the 3′ end of the S gene, within the ORF 3 gene region (nucleotide positions 1–200 in Fig. 4), that gave a sequence similarity of only 75.5%. This of course does not discount the possibility that a recombination event may have involved the proximal portion of the ORF 3 gene region as well. BLAST analysis of the S sequence of FRSCV MSU-1 against reported sequences only showed FRECV MSU-1, the previously described enteric ferret coronavirus strain (Wise et al., 2006), as the one with the most significant similarity to it. If recombination in the S gene did occur, giving rise to FRSCV, the donor coronavirus strain is yet to be discovered. An example of this phenomenon is type II FCoV, which originated from a double recombination between FCoV type I and canine coronavirus (CCV) (Motokawa et al., 1996; Herrewegh et al., 1998).

The main conclusion that can be reached from the present data is that there is a significant difference observed between the S genes of FRSCV MSU-1 and FRECV MSU-2. Whether this difference is associated with pathotype is not clear. In vivo experiments would be an obvious way to resolve this issue, but are hampered by the difficulty of growing either virus in cell culture. Another observation was that the partial S gene sequences from at least 3 different FRSCV strains, collected from different locales, were distinct from 3 different FRECV strains that also originated from different places (Fig. 1). Whether this pattern would hold up with a larger sample size remains to be determined. The two genotype-specific RT-PCR assays developed in this study would certainly be useful molecular tools to expand the current data.

Another proposed virulence marker in FIP strains is the 3c gene (Vennema et al., 1998). According to Pedersen (2009), mutation in

the ORF 3c gene is responsible for the FIPV biotype and that FCoVs of the enteric biotype possess an intact 3c gene. It is thought that a loss of 3c gene function enhances the internalization and replication of FIPVs in macrophages (Dewerchin et al., 2005). Pedersen (2009) explains that the mutations could either be SNPs which lead to premature stop codons or deletion mutations that significantly truncate the 3c gene product. In agreement to this are the findings of Chang et al. (2009) where mutations in the 3c gene were found in a majority (71.4%), of FIPV strains examined, while the gene was intact in 100% of analyzed FECVs. This lead to the conclusion that mutation in 3c cannot be the sole cause of FIP. In the ferret strains we analyzed, both systemic and enteric biotypes possess a single ORF 3 protein. The 3c-like protein in FRSCV MSU-1 was significantly truncated in comparison to FRECV MSU-2, prompting us to explore other systemic and enteric strains. Our findings show that the ferret coronaviruses have high sequence variability in ORF 3, even among strains of the same pathotype. A deletion in the 3c-like gene, giving rise to a truncated protein, was noted in two out of three systemic strains examined, while an intact ORF 3 was noted in the two enteric strains analyzed. Again, with the limited number of strains studied so far, it is not clear whether the mutation in gene 3 plays a role in the systemic nature of FRSCVs.

In conclusion, this study presents the first molecular comparison of two ferret coronavirus strains of different pathotypes. We provide molecular evidence showing that systemic strain, FRSCV MSU-1, is more closely related to enteric strain, FRECV MSU-2, than to any other group 1 coronavirus, in all structural and nsp genes examined. One important observation was that these two virus strains differed significantly in the S gene. It is conceivable that FRSCV and FRECV are genetically distinctive coronaviruses of ferrets, a hypothesis already supported by Brown et al. (2009) in the case of FCoVs. On the other hand, it is also possible that the in vivo mutation theory of Chang et al. (2009) and Pedersen (2009) applies to the ferret coronaviruses. Analysis of more strains of each virus pathotype is certainly needed to either substantiate or modify either of these two concepts.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2009.12.011.

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