Full-length ORF2 sequence-based genetic and phylogenetic characterization of Korean feline caliciviruses

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ABSTRACT

Feline calicivirus (FCV) is a highly infectious pathogen in cats and widely distributed worldwide with high genetic variation. Full-length open reading frame 2 of 5 from recently isolated Korean FCV isolates were sequenced and compared with those of global isolates. The results of phylogenetic analysis supported dividing global FCV isolates into two genogroups (type I and II) and demonstrated the presence of genogroup II in Korea, indicating their geographic spread in East Asia. High sequence variations in region E of the FCV isolates emphasizes that a novel vaccine needs to be developed to induce protective immunity against various FCV strains.

Keywords: Feline calicivirus; phylogenetic analysis; genogroup; open reading frame 2

INTRODUCTION

Feline calicivirus (FCV) is a highly infectious pathogen that generally causes acute, mild-to-moderate stomatitis and upper respiratory tract disease (URTD) in cats worldwide. The FCV genome is a 7.7 kb, positive-sense, single-stranded RNA molecule with three open reading frames (ORFs). ORF1 encodes a polyprotein that is proteolytically cleaved by the viral protease to release the nonstructural proteins, ORF2 encodes the major capsid protein VP1, and ORF3 encodes the minor capsid protein VP2 [1,2]. Parts of ORF2 sequences have been used to elucidate the phylogenetic relationships among FCV isolates [3,4]. Most phylogenetic analyses of nucleotide (nt) sequences from the ORF2 gene result in a “star-like” phylogeny with little statistical support for sub-species clusters. As a result, FCV has been considered to represent multiple strains belonging to a single diverse genotype [5,6]. In contrast, two recent independent studies suggested the presence of two distinct FCV genogroups, putting into question the classification of this virus [3,7]. Considering this question, the aim of this study was to determine the molecular characteristics and phylogenetic relationships of Korean FCV isolates with a comparison to global isolates and to validate the adequacy of phylogenetic classification based on full-length ORF2 sequence homologies.
Conflict of Interest
The authors declare no conflicts of interest.

Author Contributions
Conceptualization: Kim JS, Park YH; Data curation: Kim JS, Kim C; Funding acquisition: Park KT; Investigation: Kim JS, Kim C; Methodology: Kim JS, Chung HC; Project administration: Park KT; Resources: Kim JS, Kim C; Software: Kim JS, Kim C; Visualization: Kim JS, Kim C; Writing - original draft: Kim JS, Kim C, Park KT; Writing - review & editing: Chung HC, Park YH, Park KT.

MATERIALS AND METHODS

Viral RNA extraction and complementary DNA (cDNA) synthesis
Five Korean FCV isolates were recently recovered from two hospital-admitted cats with URTD and three healthy stray cats (Supplementary Table 1) [8]. Among them, two isolates were collected from hospital-admitted cats with clinical signs, including one (5 yr, strain 121) with mild nasal discharge and the other (3 yr, strain SU) with severe URTD (nasal bleeding, oral ulcer, and conjunctivitis). These adult cats were given a tetravalent vaccine (FHV-1, FCV, FPV and Chlamydia psittaci) containing FCV strain F-9 three times at 3-week intervals when they were 6 weeks of age. After these vaccinations, they had not been vaccinated. Three isolates were from healthy adult stray cats (age unknown) and they had never been vaccinated. Viral RNA was extracted from the cell culture supernatants of viral cultures using the viral DNA/RNA extraction kit (Intronbiotech, Korea). cDNA was synthesized using RNA to cDNA EcoDry Premix (Clontech, Japan, Cat no. 639546), which was used for sequencing the full ORF2 region.

Sequencing of full-length ORF2
Full-length ORF2 sequences of the five Korean isolates were obtained with three sets of primers, from a combination of five primers (ORF1-F/ORF2-R, ORF1-F/Cap1R, and Cap1F/ORF3-R; Supplementary Fig. 1 and Supplementary Table 2). Primers Cap1F and Cap1R were previously described [5]. The other three primers were newly designed for this study based on a comparison of the reference sequences retrieved from GenBank (Supplementary Table 2). Three overlapping DNA fragments were amplified by polymerase chain reaction (PCR), purified, and sequenced. The full-length ORF2 sequences were then obtained from the results of sequencing.

Phylogenetic analysis
Available sequence data of worldwide FCV isolates were retrieved from National Center for Biotechnology Information GenBank. In total, 43 full-length ORF2 sequences were obtained for phylogenetic analysis (dataset 1). Two additional datasets for partial ORF2 sequences were established according to previously reported strategies [3,7], demonstrating the existence of two genotypes among FCV isolates. Dataset 2 comprised 53 partial ORF2 sequences corresponding to amino acid (aa) residues 288–590 [3] and dataset 3 comprised 59 partial ORF2 sequences corresponding to aa 370–583 [7] (aa position numbers are based on positions in the FCV F9 strain).

Datasets 1, 2, and 3 were first tested by likelihood mapping analysis with 1,000 quartets using IQ-TREE version 1.6.11 to assess the phylogenetic information of the input alignments [9]. Maximum-likelihood (ML) trees of each dataset were constructed after automatic selection with IQ-TREE version 1.6.11. We applied 1000 ultrafast bootstrap (UTBoot) repetitions and the SH-aLRT test for each analysis. SH-aLRT and UTBoot supports were assigned to each branch of the ML trees and the tree was considered reliable when SH-aLRT ≥ 80% and UTBoot ≥ 95% [10].

Pairwise genetic distance (p-distance) analysis
P-distances within 43 full-length ORF2 nt sequences were calculated by MEGA V.7 software [11]. The results are displayed as a frequency distribution histogram of p-distance to examine the possibility of different genogroups among the tested FCV isolates [12].
RESULTS

The full-length ORF2 sequences of all five Korean isolates were successfully sequenced and deposited in the GenBank database (see Supplementary Table 1 for accession numbers). Likelihood mapping analysis of the three datasets are presented as partitioned triangular graphs (Fig. 1). The results revealed that the dataset of full-length ORF2 aa sequences (dataset 1) generated the highest percentage of the sum of the three corner regions and the lowest percentage of the central region among the datasets.

The ML phylogenetic tree based on full-length ORF2 sequences of 43 FCV isolates, including the five new Korean isolates, clearly showed two distinct clusters with high branch support values (SH-aLRT of 100 and UTBoot of 100). The five Korean isolates were also separated into two genogroups (Fig. 2). The frequency distribution of the 43 full-length ORF2 sequences using intra- and inter-genotype p-distances generated two well-bounded areas each within the range of 0.008–0.270. Intra-genotype p-distances were ≤ 0.227 and inter-genotype p-distances were ≥ 0.255 (Supplementary Fig. 2), which supports the presence of two genotypes among global FCV isolates [12]. The ML phylogenetic trees based on datasets 2 and 3 were also considered reliable with high branch support values (Supplementary Figs. 3 and 4). However, the phylogenetic analysis of dataset 3 based on nt sequences misclassified some genotype II strains as genotype I (Supplementary Fig. 4).

![Likelihood mapping analysis based on full-length ORF2 sequences (dataset 1), partial ORF2 sequences corresponding to amino acid (aa) 288–590 (dataset 2), and partial ORF2 sequences corresponding to aa 370–583 (dataset 3). A, B, and C show the results based on nucleotide sequences of datasets 1, 2, and 3, respectively. D, E, and F show the results based on amino acid sequences of datasets 1, 2, and 3, respectively. ORF, open reading frame.](https://vetsci.org)
The sequence comparison among 59 ORF2 partial sequences including the five new Korean isolates showed distinct variations at 3 aa positions between the two genogroups. Genotype I isolates had Asn/Asp, Ala, and Gly residues, whereas genogroup II isolates possessed Lys, Val, and Ser residues at aa positions 377, 539, and 557, respectively (Figs. 2 and 3). In addition, high sequence variability among the FCV isolates including five Korean isolates was observed in the region of the linear B-cell epitope and in the 5′ HVR of region E, compared with the vaccine strains of FCV (F9, F4, and 2024; Fig. 3).

**DISCUSSION**

Owing to the high variability in nt sequences among FCV variants, it has been difficult to generate high-quality PCR products of the ORF2 gene for sequencing [5,6,13]. In this study, the full length ORF2 of five Korean isolates was successfully sequenced using strategically designed novel primer sets binding to conserved regions of ORF1, ORF2, and ORF3 (Supplementary Fig. 1 and Supplementary Table 2). In partitioned triangular graphs generated by likelihood mapping analysis (Fig. 1), the three corner regions represent the well-resolved phylogeny (informative) and the central triangle represents a star-like evolutionary pattern (uninformative). The three rectangles represent a partly resolved phylogeny (partly informative). In general, a good dataset should show a high percentage of the sum of the
In our analysis, aa sequences were more informative than nt sequences, and the dataset of full-length ORF2 aa sequences was demonstrated to be the best fitting model in topology analysis among the three datasets of nt or aa sequences.

The ML phylogenetic tree based on full-length ORF2 sequences clearly showed two distinct clusters in global FCV isolates including the five Korean isolates. To date, FCV isolates belonging to genotype II have only been reported in Japan and China [3,7]. Therefore, this study demonstrates the presence of both genotype I and II isolates in Korean cats and represents the first report of the isolation of genotype II strains from stray cats. The limited geographical distribution of FCV genotype II isolates in Japan and China has been attributed to the regional proximity of the two countries [3]. Korea is geographically located between Japan and China, which can explain the spread of genotype II strains within the cat population of Korea. Thus, worldwide genotype II FCV strains have been identified only in three adjacent countries in East Asia with no evidence of further spread to other distant continents. These results suggest that genotype II strains are only spreading and circulating locally as endemic strains of East Asia.

Previous studies identified three distinct aa residues (at positions 377, 539, and 557) between genogroup I and II isolates [3,7]. The sequence analysis of the five Korean isolates showed the same results except for isolates 88 and SU (both belonging to genotype I). Isolates 88 and SU had a new variation at aa 377, specifically Asp instead of Asn (Figs. 2 and 3). This is the first report of this variant in the genotype I isolates.
ORF2 encodes the major capsid protein VP1 and can be divided into six regions (A–F) based on sequence conservation [1]. Region E has been proposed as an important immunological component owing to the presence of linear B-cell epitopes [15], and the Asn-Gly-Thr sequence (aa 439–441) in the 5′ hypervariable region (HVR) of region E has strong immunogenicity [16]. Therefore, the high sequence variability detected in the region of the linear B-cell epitope and in the 5′ HVR of region E among the FCV isolates (Fig. 3) suggests that immunization with a single vaccine strain should be inefficient for protection against FCV infections.

In summary, we further validated the adequacy of classification of FCV strains into genotypes I and II based on phylogenetic analysis of full-length and partial ORF2 sequences of 43 FCV isolates, including five new Korean isolates. Importantly, this study demonstrates the presence of genotype II isolates in Korea, which extends the geographic range from only China and Japan, suggesting that genotype II strains might be endemic strains circulating and evolving only in the limited geographical regions of East Asia. The high sequence variation of the immunogenic components of FCV isolates detected in this study suggests that the current vaccine strains might not be sufficient to protect against infections of variable circulating FCV strains in the cat population, warranting further efforts to develop a novel vaccine for cross-protection.

**SUPPLEMENTARY MATERIALS**

**Supplementary Table 1**  
Feline calicivirus isolates used in this study  
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**Supplementary Table 2**  
Primers used for ORF2 sequencing of FCV isolates  
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**Supplementary Fig. 1**  
Schematic presentation of the full-length ORF2 sequencing strategy. PCR fragments 1 and 2 were purified and directly sequenced with the amplifying primers corresponding to the fragments. PCR fragment 3 was cloned into a plasmid and then sequenced. The full-length ORF2 sequence of each FCV isolate was recovered from the three sequencing datasets.  
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**Supplementary Fig. 2**  
Frequency distribution of pairwise genetic distances (p-distances) among 43 ORF2 nucleotide sequences. Intra-genotype p-distances and inter-genotype p-distances formed two respective well-bounded areas within a range of 0.008–0.270, although these areas were not completely separated.  
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Supplementary Fig. 3
Phylogenetic tree of FCV isolates based on the partial ORF2 sequence corresponding to amino acids 288–590. A total of 1000 ultrafast bootstrap (UTBoot) repetitions and the SH-aLRT test were performed to construct each maximum-likelihood tree; the tree was considered to be reliable if SH-aLRT ≥ 80% and UTBoot ≥ 95%. Closed diamonds mark FCV isolates recovered in this study, and open diamonds mark FCV isolates previously classified into genotype II [3,7].

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Supplementary Fig. 4
Phylogenetic tree of FCV isolates based on the partial ORF2 sequence corresponding to amino acids 370–583. A total of 1000 ultrafast bootstrap (UTBoot) repetitions and the SH-aLRT test were performed to construct each maximum-likelihood tree. The tree was considered to be reliable if SH-aLRT ≥ 80% and UTBoot ≥ 95%. *SH-aLRT%/UTBoot%. Closed diamonds mark FCV isolates recovered in this study, and open diamonds mark FCV isolates previously classified into genotype II [3,7].

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