# Molecular Characterization of the FCoV-like Canine Coronavirus HLJ-071 in China

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## Abstract

# Background

According to differences in antigens and genetic composition, canine coronavirus (CCoV) consists of two genotypes, CCoV-Ⅰ and CCoV-Ⅱ. Recently, CCoVs with mutant variations have been found to be pantropic and pathogenic in dogs.

# Results

In this study, we isolated a CCoV, designated HLJ-071, from a dead 5-week-old female Welsh Corgi with severe diarrhea and vomiting. Sequence analysis suggested that HLJ-071 bears a complete ORF3abc when compared with classic CCoV isolates (1–71, K378 and S378). In addition, a variable region was located between the S gene and the open reading frames (ORF) 3a gene, in which HLJ-071 has a deletion of 104 nucleotides (nts) when compared with classic CCoV strains 1–71, S378 and K378. Phylogenetic analysis based on the S gene and complete sequences showed that HLJ-071 is closely related to Feline Coronavirus (FCoV) II. Recombination analysis suggested that HLJ-071 originated from the recombination of FCoV 79-1683, FCoV DF2 and CCoV A76. Finally, cell tropism experiments suggested that HLJ-071 is able to replicate in canine macrophages/monocytes.

# Conclusion

The present study involved the isolation and genetic characterization of a variant CCoV strain. The spike protein and ORF3abc of CCoV might play a key role in viral tropism, which could affect replication in monocyte/macrophage cells. This will provide essential information for further understanding the evolution of CCoV in China.


## Background

COVID-19 is once again bringing coronaviruses back into focus. Coronaviruses, which belong to the family Coronaviridae, order Nidovirales, are single-stranded positive-sense RNA viruses, which have been widely detected in wild animals [1], domesticated animals [2, 3], humans [4]and pets [5].

Currently, coronaviruses can be divided into four subfamilies, named Alpha, Beta, Gamma and Delta [6]. Canine coronavirus (CCoV) is a member of the alpha subfamily of coronaviruses which, based on the spike protein gene, has been divided into two distinct genotypes, CCoV I and CCoV II [7, 8], both of which are distributed widely [9–14]. Two different subtypes, CCoV IIa and CCoV IIb, have been found in dogs. CCoV IIa has served as the classic CCoV strain; it causes mild enteritis in young dogs [15]. CCoV IIb emerged because of homologous recombination between the transmissible gastroenteritis virus of swine (TGEV) and CCoV IIa strains [16]; it causes acute gastroenteritis and the virus can be detected in the

intestines and other internal organs [16]. An intermediate virus, CCoV-A76, which possess a distinct spike which confers pathogenicity and is the result of recombination between CCoV I and CCoV II, has been detected [17].

Recently, more virulent CCoV strains have been reported without obvious coinfections [18–20]. In these infections, variant viruses with novel recombinant and deletion forms have been detected in the intestines and other organs, resulting in new pathogenic viruses [21, 22]. In addition, HLJ-073, with deletion of ORF3abc, has altered cell tropism and can replicate effectively in canine macrophages/monocytes and human myeloid leukemia mononuclear cells (THP-1 cells) [21]. Therefore, CCoV is of concern for public health.

In this study, we isolated the HLJ-071 strain of CCoV from canine fecal samples in China. To better understand the genetic characterization of a variant CCoV strain, the complete genome sequence, phylogenetic tree and cell tropism were investigated. This will provide essential information for further understanding the evolution of CCoV in China.

# Methods

### Clinical case

During the summer of 2015, a 5-week-old female Welsh Corgi with severe diarrhea and vomiting was submitted after death for laboratory investigation. Post-mortem examination of the dog showed hemorrhagic enteritis and hemorrhages on the surface of the lungs.

### Isolation and purification

Rapid diagnosis kits were employed to identify general canine viral pathogens, including canine distemper virus (CDV), canine parvovirus (CPV), canine adenovirus-1 (CAV-1), CAV-2 and CCoV (Bionote, Hwaseong-si, Gyeonggi-do, South Korea). The primers P-F and P-R were employed as described [21].

Crandell feline kidney (CrFK) cells were grown in D-MEM supplemented with 10% fetal calf serum (FCS). The fecal sample was homogenized in phosphate-buffered saline (PBS) and centrifuged at 3,000 *g* for 15 min. Following this, the supernatant was filtered through a 0.22-μm pore size filter and inoculated into CrFK cells, which had formed confluent monolayers. When the sample was passaged three times, cytopathic effects (CPE) were observed. After three rounds of purification by plaque assay [23], the purified virus was titrated and harvested by one cycle of freezing and thawing, and aliquots were stored at −80°C.

### Electron microscopy

The electron microscopy protocol for negative-stain and thin-section examination was described previously [24, 25].

### Isolation and culture of canine blood monocytes

Canine blood monocytes were isolated following a previously described protocol [26]. Briefly, canine blood monocytes were isolated from five specific-pathogen-free (SPF) dogs. The blood mononuclear cells were purified on Histopaque-1077 (Sigma-Aldrich), and then seeded in a 24-well dish and cultured at 37 °C with 5% $CO_2$. After 24 h, nonadherent cells were removed and washed twice with PBS buffer.

### Indirect immunofluorescence assay (IFA)

The IFA was conducted using a standard procedure. Briefly, mononuclear cells were inoculated with CCoV isolates at a multiplicity of infection (MOI) of 1 for 48 h and the supernatant removed; after washing with PBS, the infected cells were fixed with paraformaldehyde (4%) for 30 min. After blocking with 2% BSA for 2 h at 37 °C, the cells were incubated with N protein polyclonal antibodies (1:400) for 1 h at 37 °C [27], followed by a fluorescein isothiocyanate-conjugated goat anti-mouse antibody against immunoglobulin G (1:1000; Abcam, UK). The CCoV-N polyclonal antibody was prepared in our laboratory [21].

### Genome sequencing and phylogeny analysis

Fourteen pairs of primers were designed, based on the conserved regions of CCoV strain HLJ-073 [21]. The RNA extraction and cDNA synthesis were performed as previously described [24].

Sequence data were assembled and analyzed using Clustal X software (1.83), Vector 10 and DNASTAR. Phylogenetic trees based on the complete sequences and the spike proteins were produced using the neighbor-joining (NJ) method with the Kimura two-parameter model in molecular evolutionary genetics analysis (MEGA) software (version 4.0). The support for the tree nodes was calculated with 1,000 replicates. All of the sequence information were listed in Table 1. Simplot 3.5.1 was used to evaluate the recombination events between the reference CCoV and FCoV strains. The HLJ-071 sequence obtained in this study was assembled and submitted to the GenBank database under accession number KY063616.

## Results

# Viral isolation and identification

The results of the colloidal gold diagnostic reagent and PCR confirmed that the fecal sample was CCoV-positive; the sample was negative for CPV, CAV and CDV (data not shown). After inoculation of CrFK cells with samples and three serial passages, one CCoV isolate, designated HLJ-071, was obtained from the fecal samples; CPE were found in the CrFK cells 3–5 days post-inoculation, with rounding and detachment of cells into the medium (Fig. 1A, 1B). The titre of HLJ-071 was $10^{7.5}$ median tissue culture infective dose ($TCID_{50}$)/mL in CrFK cells. Electron microscopy showed that the virus had a circular shape with petal-shaped protrusions, and had a diameter of about 150 nm (Fig. 1C). Ultra-thin sections of infected CrFK cells displayed typical virus particles in the cytoplasm (Fig. 1D).

# Full-length nucleotide sequence and phylogenetic analysis

The complete genome sequence of HLJ-071 was assembled and comparative analysis with other canine coronavirus was performed. The full genome of HLJ-071 was amplified using the 14 pairs of primers referenced to HLJ-073. The complete sequence of HLJ-071 was 29,319 nucleotides (nts) in length, including the 5'non-translated region (NTR)-ORF1-S-ORF3abc-E-M-N-ORF7ab-3'poly A tail. The 5'portion NTR of the genome contained a 230-nt NTR, ORF1a (231–12,287) and ORF1ab (231–20,057). Four structural proteins, S, E, M and N, were found to be encoded by ORF S (20,284–24,648 nt), ORF E (25,826–26,074 nt), ORF M (26,055–26,876 nt) and ORF N (26,889–28,037 nt). Five non-structural protein-encoding genes were ORF3a (24,712–24,948 nt), ORF3b (24,893–25,108 nt), ORF3c (25,105–25,860 nt), ORF7a (28,042–28,347 nt) and ORF7b (28,352–28,993 nt).

Sequence analysis suggested that there was an entire ORF3abc with 1,149 nts when compared with classic CCoV strains 1–71 and K378. However, a variable region was located between the S gene and ORF 3a gene, in which HLJ-071 had a deletion of 104 nts when compared with classic CCoV strains 1–71, S378 and K378 (Fig. 2). In addition, variant CCoV strains, feline enteric coronavirus (FECV), feline infectious peritonitis virus (FIPV) and TGEV also bear a deletion, to varying degrees, when compared with classical CCoVs. A highly conserved core sequence (CS), 5'-CUAAAC-3', is present in HLJ-071 and variant CCoV, FECV, FIPV and TGEV strains. There is a C to T mutation in classical CCoVs, resulting in a different CS, 5'-UUAAAC-3'. All of these findings suggest that the transcription of ORF3abc is influenced by these variable regions.

In the phylogenetic analysis based on complete sequences, HLJ-071 fell into the FCoV cluster, close to TN-449, HLJ-073 and A76. The complete spike protein did not cluster with either type I or type II CCoVs, and was related to FCoV WSU 79-1683. In addition, analysis of the S1 (receptor-binding) domain showed that it clustered closely with FCoV 79-1683 and HLJ-073, while the S2 (fusion) domain clustered with CCoV IIb 174/06 and FCoV WSU79-1683 (Fig. 3). The occurrence of recombination was detected in HLJ-071, involving CCoV A76, FCoV 79-1683 and DF2, which has led to the emergence of a new genotype of FCoV-like CCoVs (Fig. 4).

# Cell tropism of HLJ-071

Previous studies showed that ORF3abc deletion in canine and feline coronavirus alters the cell tropism [26, 27]. To investigate further the *in vitro* growth characteristics of HLJ-071, canine monocytes were inoculated with HLJ-071 and HLJ-073 at an MOI of 1. The results showed that HLJ-073 can replicate efficiently in canine monocytes; however, HLJ-071 could replicate only poorly in these cells (Fig. 5). Furthermore, the titers of HLJ-071 and HLJ-073 were determined at 24 h.p.i. in CrFK cell lines and canine monocytes. The results showed that the growth characteristics of HLJ-071 were similar to those of HLJ-073 and reached $10^{7.5}$ $TCID_{50}$/mL at 24 h p.i. in CrFK cells (Fig. 6A). However, the titer of HLJ-071 peaked at $2\times10^{1.6}$ $TCID_{50}$/mL at 6 h p.i., showing significantly slower growth when compared with the titer of HLJ-073, which reached $10^{3.5}$ $TCID_{50}$/mL at 36 h.p.i. (Fig. 6).

# Discussion

Generally, CCoV causes intestinal infections, resulting in viral enteritis and diarrhea in dogs. In recent decades, however, an increasing number of pantropic strains have been reported to cause systemic infections with multiple organ damage [28]. The mechanism for the change in tissue tropism of CCoVs from enteropathogenic to systemic infection is unknown.

In this study, we isolated a CCoV, HLJ-071, from a dead 5-week-old female Welsh Corgi without apparent coinfections. Phylogenetic analysis based on the complete sequence suggested that HLJ-071 is close to FCoV II and distinct from other CCoV I and II strains. In terms of the major structural protein spike protein, HLJ-071 was closely related to FCoV WSU79-1683 and domestic strain HLJ-073, different from other Chinese strains B135/JS/2018, B194/GZ/2019, B639/ZJ/2019, B203/GZ/2019 and B447/ZJ/2019. HLJ-071 recombining with CCoV A76, FCoV 79-1683 and DF2, formed a unique cluster with HLJ-073 and TN-449 between the classical CCoVs and FCoVs. All of these findings suggest that domestic CCoV strains were derived from different ancestors and have co-circulated in China and undergone evolution.

The cell tropism of FCoVs has been well investigated. Previous studies have indicated that the spike protein and ORF3abc play a crucial part in infection of macrophages by FCoVs [26, 29]. Feline infectious peritonitis viruses (FIPV) bearing partial deletion of ORF3abc are able to replicate in cells of the monocyte/macrophage lineage and then disseminate to the organs, causing systemic infections [30]. In contrast, feline enteric coronavirus (FECV) with entire ORF3abc primarily replicates in enterocytes and is unable to replicate in cells of the monocyte/macrophage lineage. Among CCoVs, a systemic infection involving CCoV CB/05, with a partial deletion of ORF3b and a FCoV-like spike protein, was found in 2005 [31]. We speculate that this stain could replicate in monocyte/macrophage cells, although no data were available from viral tropism experiments. Furthermore, we have isolated a CCoV strain, HLJ-073, bearing a 350-nt deletion in ORF3abc and a FCoV-like spike protein, and reported that it can replicate efficiently in canine monocyte/macrophage cells and human THP-1 cells [21]. In this study, HLJ-071, bearing the entire ORF3abc and FCoV spike protein, could replicate only weakly in canine monocyte/macrophage cells when compared with HLJ-073, which was distinct from the cell tropism of FECV. These findings suggest that 1) both HLJ-071 and HLJ-073 have a common feature, the acquisition of macrophage tropism; 2) deletion of ORF3abc can enhance replication in monocyte/macrophage cells, as shown by comparison of HLJ-071 and HLJ-073.

Previous studies have indicated that the 5' and 3' flanks of the transcription regulatory sequences (TRSs) of TGEV influence transcription levels (accumulation) and protein expression [32]. Interestingly, when comparing classic CCoVs, variant CCoVs, FECV, FIPV and TGEV, sequence analysis suggests that there is a variable region forward of the TRS, between the end of spike gene and ORF3a gene, which may influence the level and stability of RNA transcription and gene expression. Among the CCoV, FCoV and TGEV, a C to T mutant only found in the conserved CS of classic CCoVs K378 and 1–71, which may speculate that the transcription of subgenome was affected. Both of these findings suggest that, except for the function of the ORF3abc protein of CCoV, the variable region may also contribute to alterations in cell tropism of CCoV. Further experiments are needed to investigate the mechanisms involved between the transcription of the subgenome and the function of ORF3abc in viral tropism.

# Conclusion

In this study, we indicated that: 1) CCoV HLJ-071 is closely related to FCoVs and shows recombination between CCoVs and FCoVs, indicating that CCoVs have undergone a rapid evolution in China. 2) The cell tropism of CCoVs may be correlated with the function of ORF3abc and transcription of the subgenome.

# Abbreviations

Feline Coronavirus (FCoV); Canine coronavirus (CCoV); transmissible gastroenteritis virus (TGEV); human myeloid leukemia mononuclear cells (THP-1 cells); canine distemper virus (CDV); canine parvovirus (CPV); canine adenovirus-1 (CAV-1); Crandell feline kidney (CrFK); fetal calf serum (FCS); cytopathic effects (CPE); neighbor-joining (NJ); molecular evolutionary genetics analysis (MEGA); core sequence (CS); Feline infectious peritonitis viruses (FIPV); transcription regulatory sequences (TRSs); multiplicity of infection (MOI); median tissue culture infective dose (TCID50)

# Declarations

### Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of Yibin University, Yibin, China, according to the OIE standards for use of animals in research and education. No animals were sacrificed specifically for this study. The dog was collected by the veterinary hospital. The owner of dog took the initiative to send the sick animal to the veterinary hospital for treatment. When the dog's condition worsens until death, and with the client’s consent for further research, the body of the dogs were sent to University of Yibin. We reported the results of death of the dogs to the veterinary hospital according to the diagnostic findings, and we were not involved in the collection of samples.

### Consent for publication

Not applicable.

### Availability of data and materials

DNA sequences obtained in this study have been submitted to the GenBank database (accession number: KY063616).

### Competing interests

The authors declare no competing interests.

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preparation of the manuscript.

### Authors' contribution

HXL designed the study, TZG, ZMM, DY and GDD performed the experiments. TZG drafted the manuscript. GP, CF and HXL revised the draft. All authors revised and approved the paper for publication.

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# Table

Table 1. Source of sequences used in the experiment

| Isolate | Type | Accession no. | Origin | Collection date |
|---|---|---|---|---|
| CB/05 | CCoV IIa | KP981644 | ITA | 2005 |
| 1-71 | CCoV IIa | JQ404409 | GER | 1971 |
| K378 | CCoV IIa | KC175340 | USA | 1978 |
| TGEV-HX | / | KC962433 | CHN | 2013 |
| BGF-10 | CCoV IIa | AY342160 | UK | 2002 |
| INSAVC-1 | CCoV IIa | D13096 | UK | 1992 |
| A76 | CCoV II | JN856008 | USA | 1976 |
| Purdue | / | DQ811789 | USA | 2011 |
| 23/03 | CCoV I | KP849472 | ITA | 2004 |
| Black | FCoV I | EU186072 | USA | 1980 |
| WSU79-1683 | FCoV II | JN634064 | USA | 1979 |
| TN449 | CCoV IIa | JQ404410 | USA | 1980s |
| DF2 | FCoV II | JQ408981 | USA | 1981 |
| 79-1146 | FCoV II | DQ010921 | USA | 1979 |
| 171 | CCoV IIa | KC175339 | GER | 1971 |
| HCOV 229E | / | AF304460 | / | 1963 |
| S378 | CCoV IIa | KC175341 | USA | 1978 |
| GZ43 | / | EF192155 | CHN | 2003 |
| KUK-HL | FCoV II | AB781789 | JPN | 2014 |
| 450/07 | CCoV IIb | GU146061 | ITA | 2012 |
| FC1 | CCoV IIa | AB781790 | JPN | 2014 |
| M91-267 | FCoV II | AB781788 | JPN | 2013 |
| RM | FCoV I | FJ938051 | USA | 2002 |
| NTU156 | FCoV II | GQ152141 | CHN | 2007 |
| NTU336 | CCoV II | GQ477367 | CHN | 2008 |
| 174/06 | CCoV II | EU856362 | ITA | 2006 |
| 430/07 | CCoV II | EU924790 | ITA | 2007 |
| 68/09 | CCoV IIb | HQ450377 | GRE | 2009 |
| 341/05 | CCoV II | EU856361 | ITA | 2005 |

| | | | | |
|---|---|---|---|---|
| 66/09 | CCoV IIb | HQ450376 | GRE | 2009 |
| 119/08 | CCoV II | EU924791 | ITA | 2008 |
| UU16 | FCoV I | FJ938058 | NED | 2007 |
| UU21 | FCoV I | HQ012369 | NED | 2007 |
| UU23 | FCoV I | GU553362 | NED | 2007 |
| UU2 | FCoV I | FJ938060 | USA | 1993 |
| CATDAY28 | FCoV I | KU215428 | BEL | 2013 |
| UCD11a | FCoV I | FJ917519 | USA | 2008 |
| DM95/2003 | FbCoV | EF192156 | CHN | 2007 |
| HCM47 | CCoV II | LC190907 | VNM | 2019 |
| B639 ZJ 2019 | CCoV II | MT114538 | CHN | 2019 |
| B194 GZ 2019 | CCoV II | MT114543 | CHN | 2019 |
| B203 GZ 2019 | CCoV II | MT114542 | CHN | 2019 |
| B447 ZJ 2019 | CCoV II | MT114540 | CHN | 2019 |
| B135 JS 2018 | CCoV II | MT114544 | CHN | 2019 |

# Figures

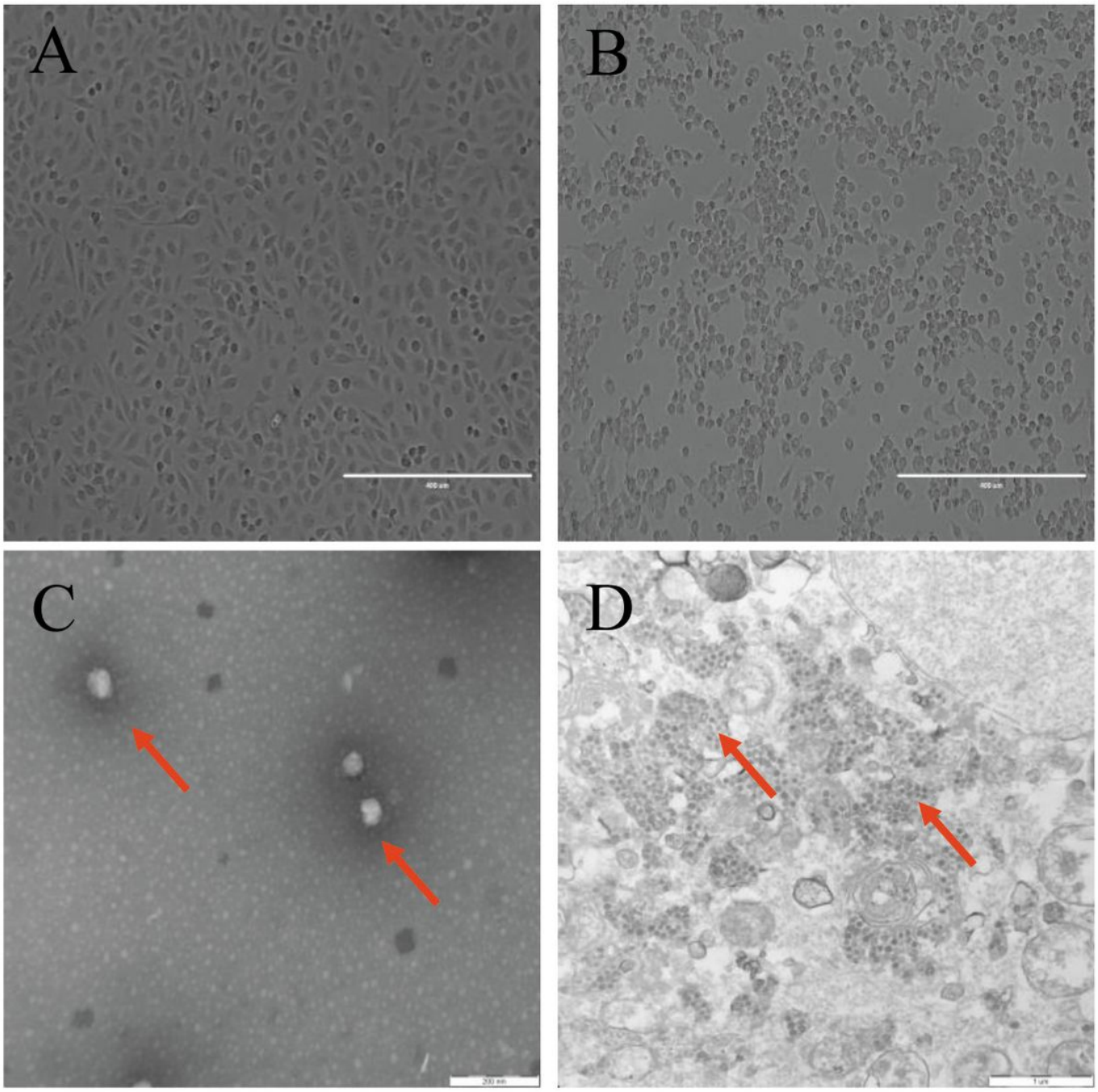
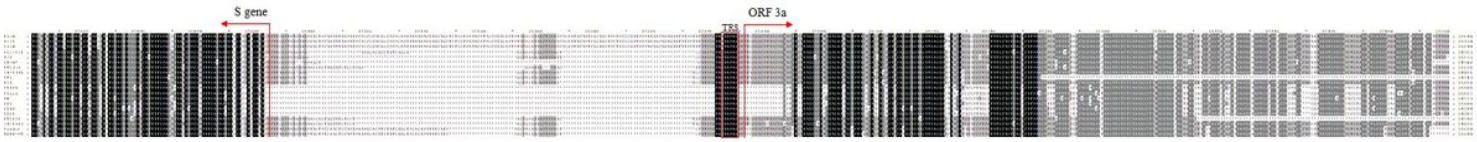


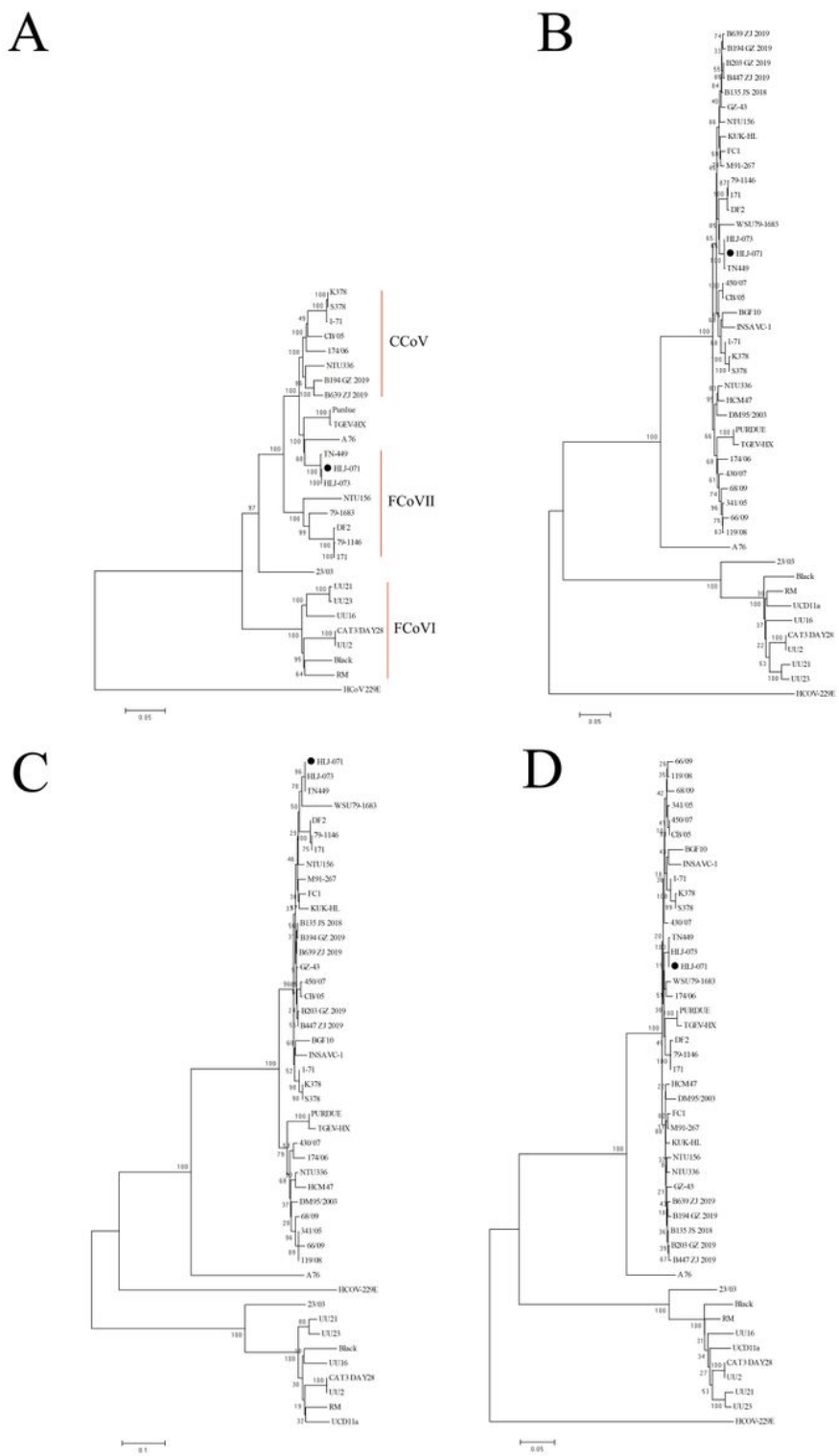

**Figure 1**

(A) Control (uninfected) CrFK cells. (B) Cytopathic effect (CPE) induced by HLJ-071 in CRrFK cells. (C) Electron micrograph of HLJ-071 negatively stained with 2% phosphotungstic acid. The scale bar represents 200 nm. (D) Ultra-thin sections of CrFK cells infected with HLJ-071 display the typical particles in the cytosol.



## Figure 2

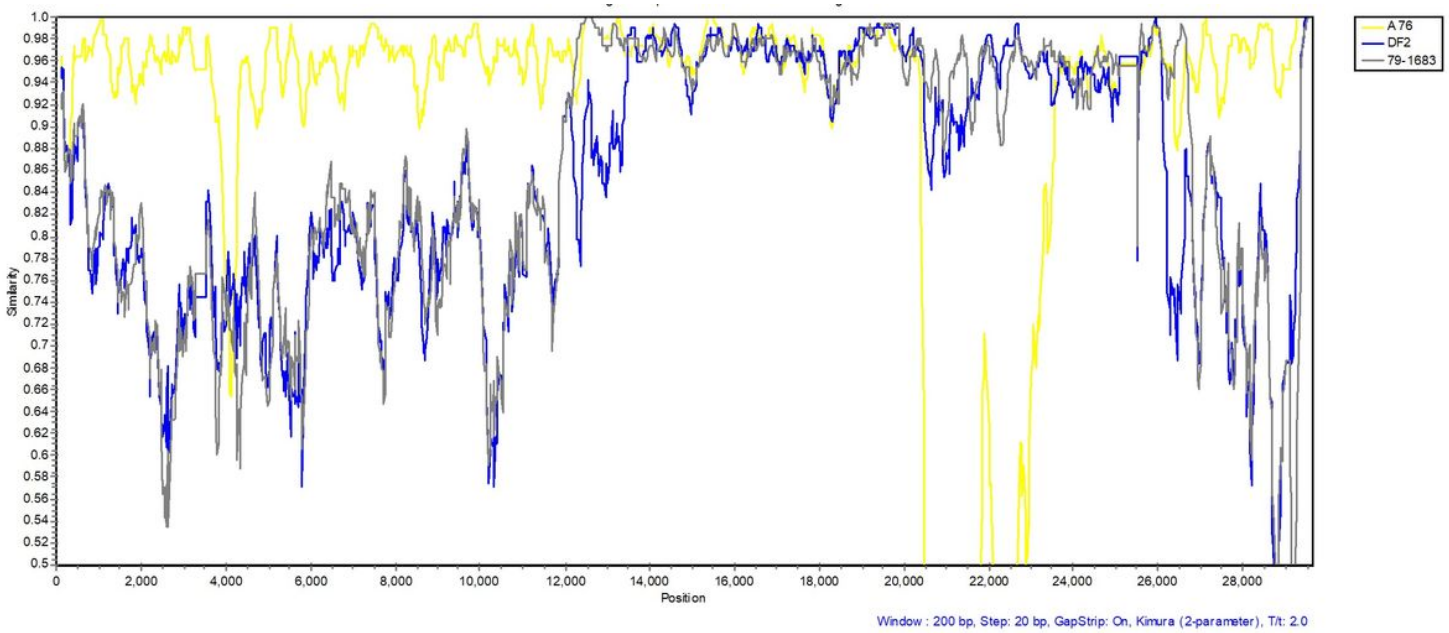
Multiple sequence alignment of the partial S and ORF3a of CCoV strains.




## Figure 3

Phylogenetic analysis of the complete sequences, spike protein (S), S1, and S2 genome regions of HLJ-071. Neighbor-joining was used for construction of the phylogenetic tree, with bootstrap values of 1000

replicates shown at the branches. The scale bar represents the p-distance.




## Figure 4

Similarity plot of the complete nucleotide sequences of CCoV HLJ-071 and three reference CCoV strains, FCoV DF2, FCoV WSU79-1683 and CCoV A76. The other parameters used included the Kimura (two-parameter) distance model, 2.0 Ts/Tv ratio, neighbor-joining tree model, and 1000 bootstrap replicates.

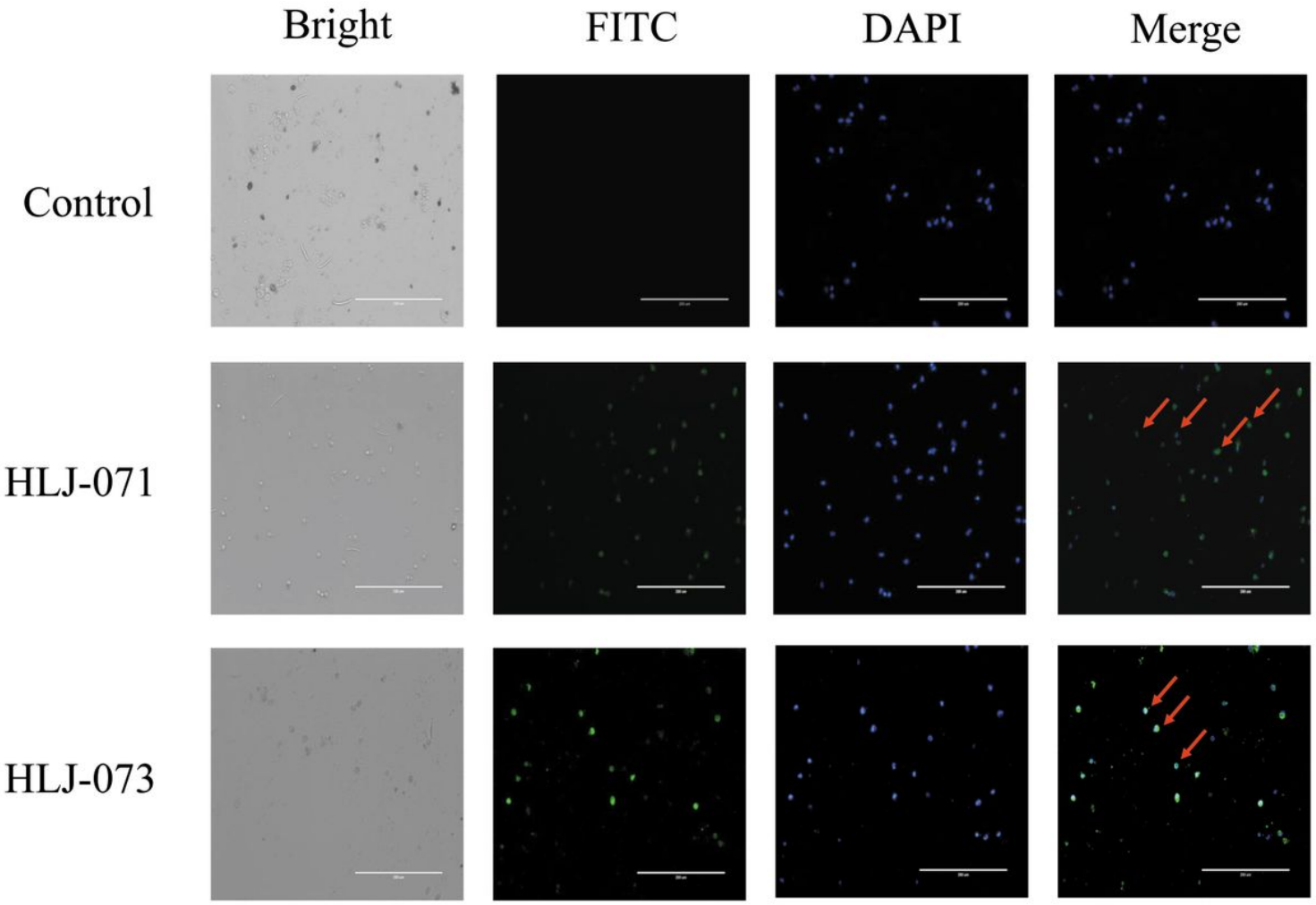



**Figure 5**

Indirect immunofluorescence detection of CCoVs in canine macrophages/monocytes. The cells were infected with HLJ-071 or HLJ-073 (MOI = 1) and detected using CCoV N protein-positive serum.

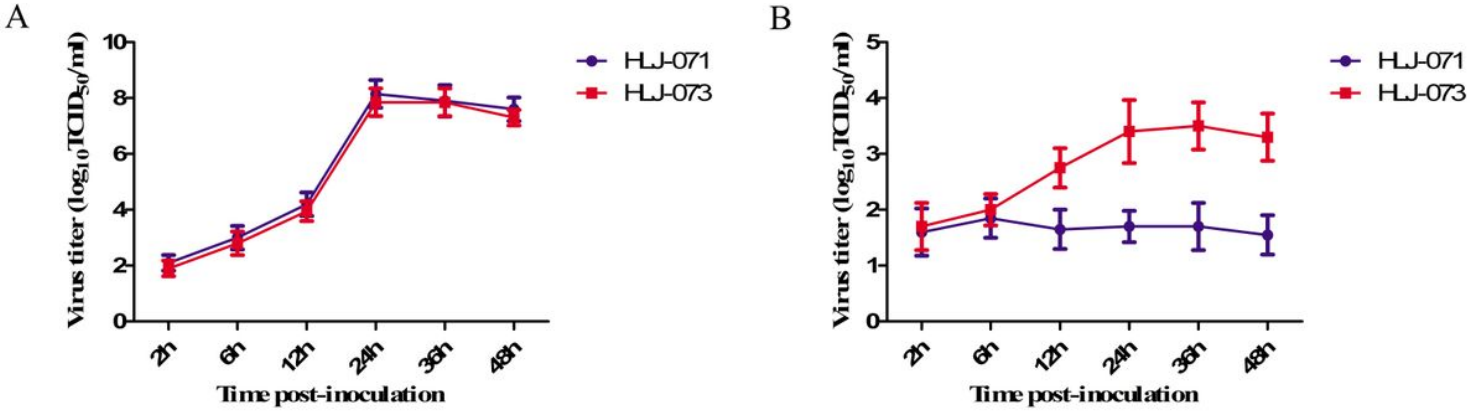



**Figure 6**

Replication dynamics of HLJ-071 and HLJ-073. Growth kinetics of HLJ-071 and HLJ-073 after infection of CrFK (A) and canine macrophage/monocyte (B) cells (MOI of 1). The titers are given as the means

from triplicate experiments (log 10 TCID 50/ml); error bars represent standard deviations.