



Molecular characterization of HLJ-073, a recombinant canine coronavirus strain from China with an ORF3abc deletion

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Abstract

Canine enteric coronaviruses (CCoVs) are important enteric pathogens of dogs. CCoV with different variations are typically pantropic and pathogenic in dogs. In this study, we isolated a CCoV, designated HLJ-073, from a dead 6-week-old male Pekingese with gross lesions and diarrhea. Interestingly, sequence analysis suggested that HLJ-073 contained a 350-nt deletion in ORF3abc compared with reference CCoV isolates, resulting in the loss of portions of ORF3a and ORF3c and the complete loss of ORF3b. Phylogenetic analysis based on the S gene showed that HLJ-073 was more closely related to members of the FCoV II cluster than to members of the CCoV I or CCoV II cluster. Furthermore, recombination analysis suggested that HLJ-073 originated from the recombination of FCoV 79-1683 and CCoV A76, which were both isolated in the United States. Cell tropism experiments suggested that HLJ-073 could effectively replicate in canine macrophages/monocytes and human THP-1 cells. This is the first report of the isolation of strain HLJ-073 in China, and this virus has biological characteristics that are different from those of other reported CCoVs.

Keywords Canine coronavirus · ORF3abc · Recombination · Pantropic · Viral cell tropism

Abbreviations

CCoV	Canine coronavirus
TGEV	Transmissible gastroenteritis virus
CDV	Canine distemper virus
CPV	Canine parvovirus
CAV-1	Canine adenovirus 1

Introduction

Canine coronavirus (CCoV) is a member of virus family *Coronaviridae*, order *Nidovirales*. CCoV is single-stranded positive-sense RNA viruses with a genome that is between 28 and 31 kb in length [1]. Based on antigenic and genetic relationships, CCoV consists of two distinct genotypes, CCoV-I and CCoV-II [7, 14]. CCoV was first recognized as an enteric pathogen of dogs in 1971, when it was observed to cause clinical signs including anorexia, lethargy, vomiting, and mild-to-severe diarrhea [3]. The clinical signs usually lasted 1–2 weeks and were followed by recovery, or occasionally by dehydration that led to death, mainly in puppies [6].

In recent years, researchers have reported an increasing number of cases of virulent CCoV infection in dogs and the emergence of CCoV variants with recombinant S genes. Recombination occurs when different CCoV types infect the same host simultaneously [9, 16, 20, 22]. Therefore, CCoV has become a cause of concern as an emerging pathogen in dogs. In the present study, we report the emergence and molecular characterization of an, FCoV-like recombinant CCoV-HLJ-073, which was isolated from a fecal sample from a dead dog that exhibited enteritis.

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Materials and methods

Clinical case

During the spring of 2015, a dead dog, a privately owned 6-week-old male Pekingese, was submitted for laboratory investigation. The clinical symptoms were lethargy, inappetence, fluid diarrhea, vomiting and dehydration with death after 2 days. Necropsy of the dog showed hemorrhagic enteritis and severe lesions in the liver, spleen and lung. Hemorrhages of the liver and spleen were observed on their surfaces. Multiple areas of congestion were found in the lung.

Isolation, identification, and purification of the virus

A fecal sample was collected and subjected to virological investigations. Rapid kits were employed to identify common 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 (Table S1) were employed to confirm the above results [5].

The CRFK cell line was used for virus isolation. After three rounds of purification by plaque assay [23], the virus was propagated and titrated and then harvested by one cycle of freezing and thawing. Viral RNA was extracted from the original fecal sample and from CRFK cells infected with CCoV using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Fifteen primer pairs were designed based on the conserved regions of CCoV strain TN-449 (Table S1). The RT-PCR assay was performed as described previously [13].

Electron microscopy

The protocol for electron microscopy was described previously [13].

Indirect immunofluorescence assay (IFA)

An indirect immunofluorescence assay (IFA) was conducted using a standard procedure. An anti-CCoV antibody (VMRD, WA, USA) and FITC-conjugated rabbit anti-dog IgG (Sigma, CA, USA) were used as primary and secondary antibodies, respectively. A CCoV-N polyclonal antibody was prepared as follows [15]: Briefly, the complete N gene was amplified using a forward primer (5' TTTGGATCCATG GCCAACCGGGACAACGC3') and a reverse primer (5' TTTGCGGCCGCTTAGTTCGTTACCTCATCAAT3'). Then, the products were cloned into the vector pGEX6p-1. Purified GST-N recombinant protein was used as an antigen to inject female BALB/c mice. After three immunizations, serum was collected and stored at -80 °C.

Quantitative real-time RT-PCR assay

RNA was extracted from fecal and organ samples using a previously established TaqMan-based real-time RT-PCR assay for rapid detection and quantification of CCoV RNA [10].

Detection of subgenomic (sg) ORF3abc mRNAs by RT-PCR

The primers DF2F and ORF3CR (Table S1) [2] were used to detect sg mRNAs from the ORF3abc region by RT-PCR, using a One-Step RT-PCR Kit (abm, Richmond, BC, Canada).

Sequence alignment and phylogenetic analysis

A phylogenetic tree based on the sequences of the structural proteins was constructed using the neighbor-joining (NJ) method of molecular evolutionary genetics analysis (MEGA) software (version 4.0). Bootstrap values were estimated for 1,000 replicates. Simplot 3.5.1 was used for nucleotide sequence comparison of HLJ-073 to the reference FCoV strains and CCoV strains. The HLJ-073 sequence obtained in this study was assembled and submitted to the GenBank database under accession number KY063618.

Cell tropism experiments

Canine blood monocytes originating from specific-pathogen-free (SPF) dogs were isolated [2]. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, USA). The human monocytic cell line THP-1 was provided by the National Institute for Communicable Disease Control and Prevention, Chinese Centers for Disease Control and Prevention (Beijing, China). THP-1 cells were induced to differentiate into a mature-macrophage-like state by stimulation with 50 ng of PMA per mL for 48 h before treatment.

Results and discussion

We isolated a canine coronavirus with a deletion in the ORF3abc region from a dead dog in China. Real-time RT-PCR assays were carried out to determine the viral load in the faeces, intestine, lung, liver, spleen, kidney and heart of the dead dog. All of the organs except the kidney were found to be positive for CCoV (Table S2), indicating that this strain is a pantropic CCoV strain. Next, after three rounds of plaque assay screening, a novel isolate, HLJ-073, was successfully isolated and purified. The presence

of virus particles was confirmed by electron microscopy of negatively stained samples (Fig. S2). Sequence analysis suggested that there was a 350-nt deletion in ORF3abc when compared to isolates 1-71, TN-449 and BGF-10. This deletion resulted in truncations in ORF3a (103 nt) and ORF3c (696 nt) and the complete loss of ORF3b (Fig. 1A and B). To investigate the stability of the ORF3abc region in the HLJ-073 genomic RNA, the virus was propagated in CRFK cells for nine generations. Total RNA was extracted from HLJ-073-infected CRFK cells and from the original faecal sample. Analysis by PCR confirmed that the ORF3abc deletion was present not only in the purified virus but also in the original faecal sample (Fig. S1), indicating that the deletion was not due to viral adaptation during cell culture passage. Examination of subgenomic ORF3abc mRNAs showed that the genome of HLJ-073 contained a truncated ORF3abc (Fig. 1C). Previous studies have demonstrated that this region is variable, and a natural CB/05 strain that causes fatal infections and long-lasting lymphopenia has also been

shown to contain a deletion in ORF3b [4, 12, 8]. Strains 450/07 and NA/09 are closely related to the prototype strain CB/05, which causes diarrhea and acute lymphopenia [11, 17]. Furthermore, strain BGF10 also has a deletion in the ORF3abc region [21]. These results demonstrate that the ORF3abc region may be prone to being lost during the evolution of the virus.

Recombination plays a vital role in the evolution of CCoV, allowing the emergence of new strains with altered virulence and immunogenicity. In recent years, CCoVs with different genotypes and subgenotypes have been detected. CCoV-II is the oldest genotype, which has been known since 1971 [3]. More recently, recombination between CCoV and TGEV has been detected, which has led to the emergence of CCoV-IIb isolates in which the N-terminus of the S gene is very similar to that of TGEV, while the rest of the genome resembles that of the reference CCoV-II isolate [9]. A CCoV strain (UCD-1) that had potentially recombined with TGEV was identified in the late 1990s

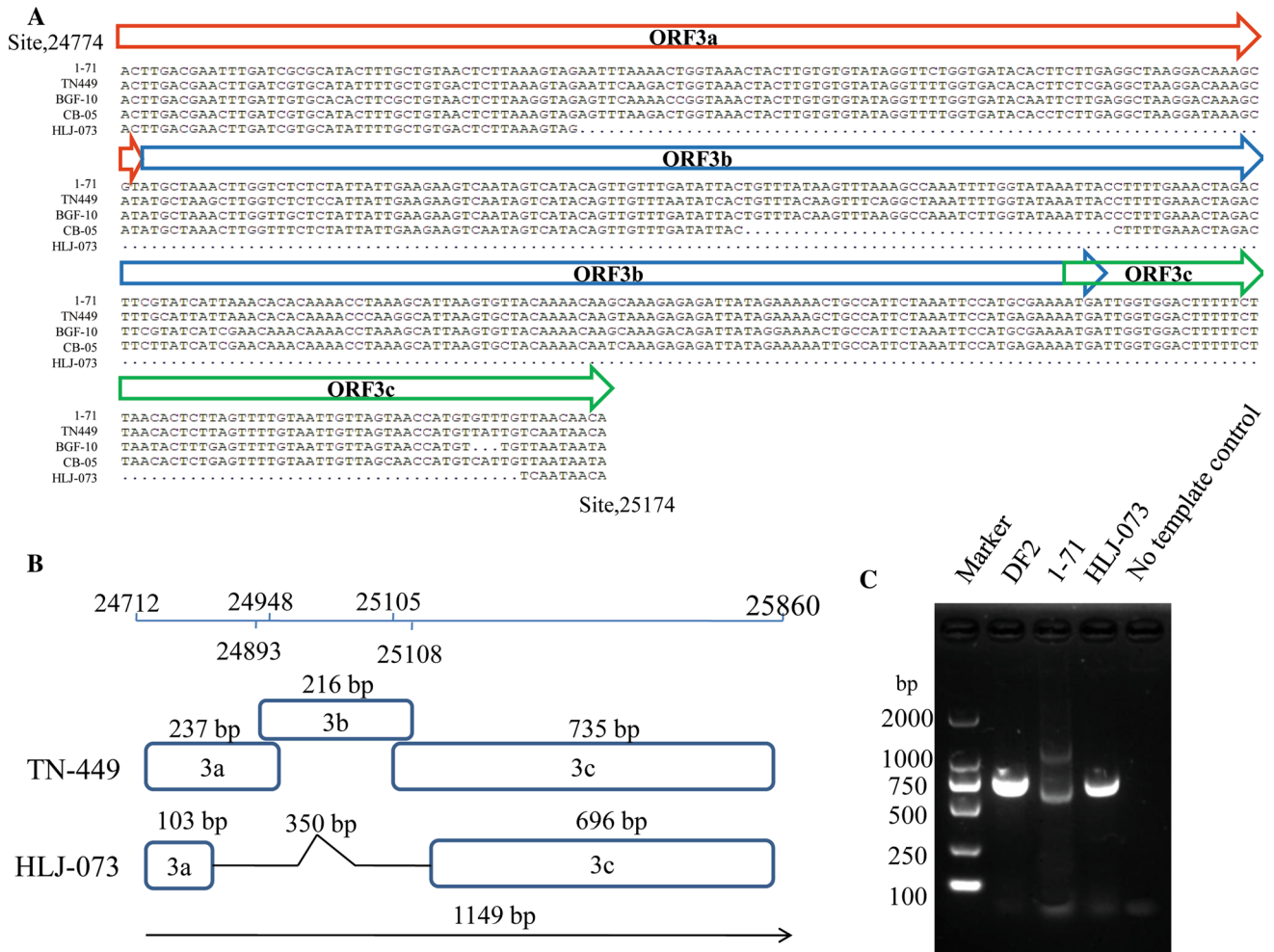


Fig. 1 (A) Multiple sequence alignment of the ORF3abc region of CCoV strains (B) Schematic diagram of ORF3a, 3b and 3c of CCoVs. (C) Identification of mutant strains by RT-PCR

[24]. Genetic analysis of several CCoV strains circulating in Italy first revealed a new canine genetic cluster in which recombination events within the S gene had increased the similarity of CCoVs to the feline homolog [19]. On the basis of their genetic relationship to FCoV-I, the new genotype was initially designated “FCoV-like CCoVs”. Recently, an additional ORF, named ORF3, located between the end of the S gene and the ORF3a gene, was also detected [14]. In this study, phylogenetic analysis based on the S protein revealed that HLJ-073 is closely related to members of the FCoV-II cluster and is distinct from CCoVs (Fig. 2A). Analysis of the S1 (receptor-binding) domain showed that it clustered closely with FCoV-II 79-1683 (Fig. 2B), while the S2 (fusion) domain clustered with CCoV-II, FCoV-II

and TGEV (Fig. 2E). The coronavirus S1 protein consists of two independent subdomains, the N-terminal domain (NTD) and the C-terminal domain (C-domain) [18]. The NTD clustered closely with CB/05 and 79-1683 (Fig. 2C), and the C-domain clustered with 79-1683 (Fig. 2D). In contrast, the M gene clustered with A76 and had a 2-amino-acid deletion compared with A76, CB/05, 23/03 (Fig. 2H), and the E gene clustered with TGEV and did not cluster with CCoV and FCoV (Fig. 2G). These data indicate that HLJ-073 is probably a recombinant of TGEV, FCoV-II and CCoV-I/II, with recombination sites located between the S, E and M genes. Simplot analysis was carried out to identify possible recombination events and breakpoints in the complete nucleotide sequence of the HLJ-073 genome. The analysis

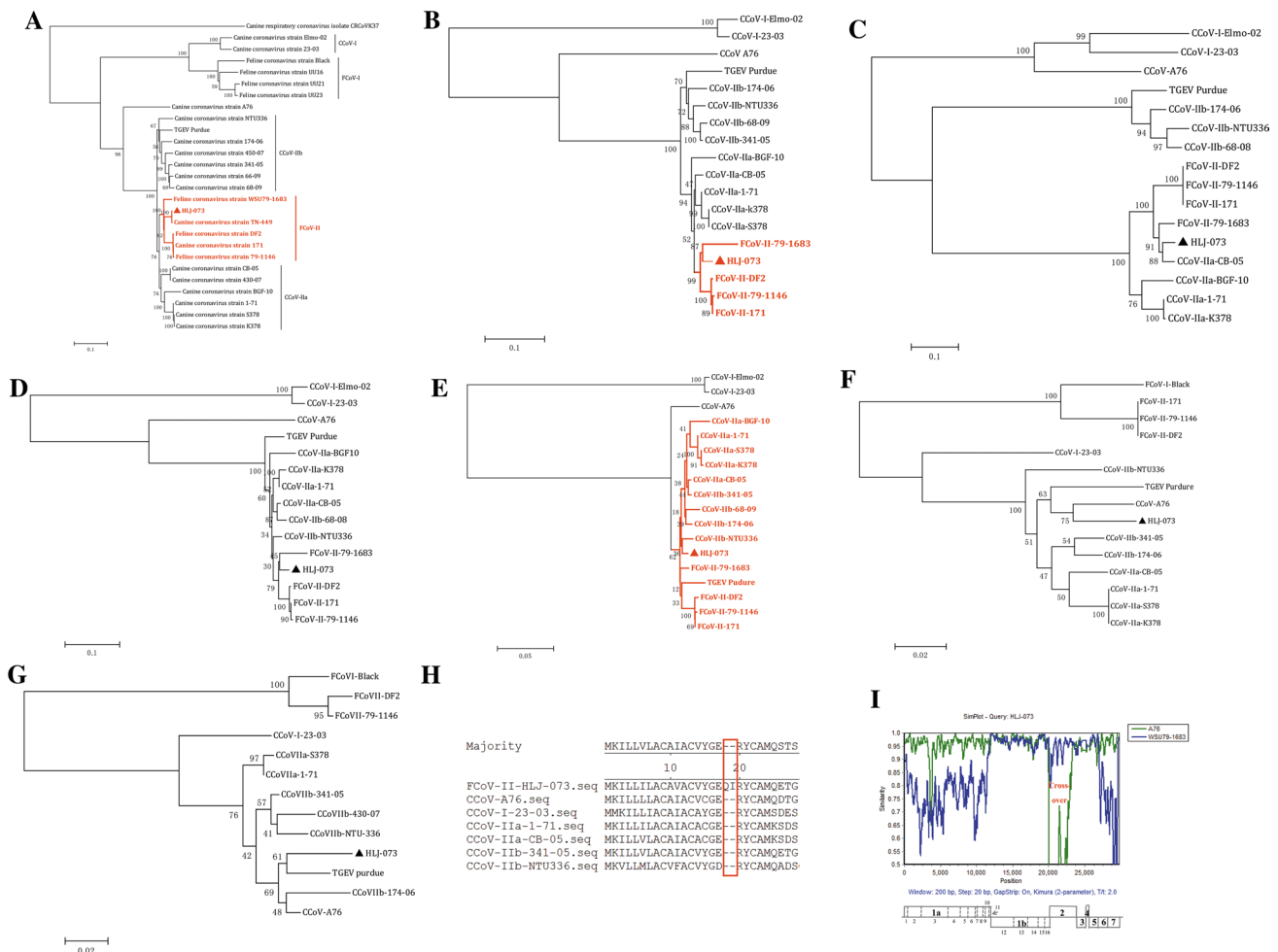


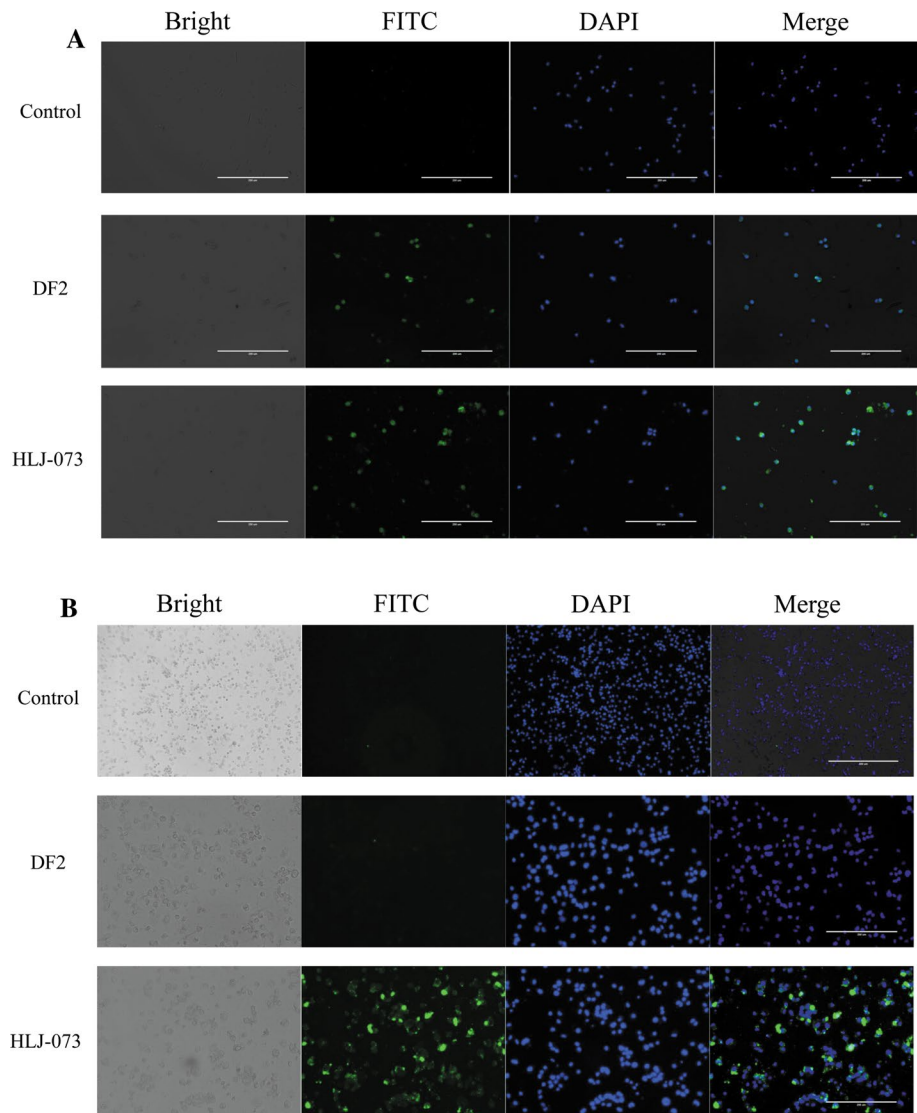
Fig. 2 (A–G) Phylogenetic analysis of the spike protein (S), S1, S1-NTD, S1-C-domain, S2, membrane (M) and envelope (E) genome regions of HLJ-073 and the most closely related strains in GenBank for which whole genome sequences were available. Neighbor-joining was used for the construction of the phylogenetic tree with bootstrap values of 1000 replicates shown at the branches. The scale bar represents the p-distance. (H) Sequence alignment of the N-terminal membrane region of CCoV strains analyzed in the present study

with CCoV strains A76, 23-03, 1-71, CB-05, 341-05, and NTU336. (I) The crossover region in the HLJ-073 genome was detected by Simplot 3.5.1. The y-axis shows the percentage of permuted trees employing a sliding window of 200 nucleotides (nt) and a step size of 20 nt. The other parameters used included the Kimura (2-parameter) distance model, 2.0 Ts/Tv ratio, neighbor-joining tree model, and 1000 bootstrap replicates

confirmed the probably occurrence of recombination in the S, E and M genes of HLJ-073, which was probably the result of recombination between FCoV 79-1683 and CCoV A76.

The ORF3abc region of feline coronavirus has been shown to affect viral cell tropism. Truncated ORF3abc of FIPV DF-2 has been shown to effectively replicate in macrophages/monocytes, while infection with FCoVs with the complete ORF3abc region was confined to the intestinal tract [2]. In the present study, we found that HLJ-073 and FIPV DF-2 could also effectively replicate in canine macrophages/monocytes (Fig. 3A). However, HLJ-073 could replicate in THP-1 cells, while DF-2 could not (Fig. 3B). These results suggest that HLJ-073 has the ability to alter its tissue tropism from the intestinal tract to systemic infection and to alter its viral cell tropism from dogs to humans, which should be regarded as a potential threat to domestic dogs. Accordingly, epidemiologic studies are required to determine whether domestic CCoV isolates have similar characteristics.

Fig. 3 Indirect immunofluorescence detection of CCoVs in canine macrophage/monocyte (A) and THP-1 cells (B). The cells were infected with HLJ-073 or DF2 (MOI = 1) and detected using CCoV N protein-positive serum



Conclusion

We report for the first time that CCoV HLJ-073, with an ORF3abc deletion, has been isolated in China. HLJ-073 represents a recombinant coronavirus with distinct host cell tropism.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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