

Novel Canine Coronavirus Isolated from a Hospitalized Pneumonia Patient, East Malaysia

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Key points: This is the first complete genome characterization of a novel canine-feline recombinant alphacoronavirus isolated from a child with pneumonia. Similar to severe acute respiratory syndrome coronavirus, this novel virus possesses some unique genetic features suggestive of recent zoonotic transmission.

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ABSTRACT

BACKGROUND During the validation of a highly sensitive pan-species coronavirus (CoV) semi-nested RT-PCR assay, we found canine CoV (CCoV) RNA in nasopharyngeal swabs from eight (2.5%) of 301 patients hospitalized with pneumonia during 2017-18 in Sarawak, Malaysia. Most patients were children living in rural areas with frequent exposure to domesticated animals and wildlife.

METHODS Specimens were further studied with universal and species-specific CoV and CCoV one-step RT-PCR assays, and viral isolation was performed in A72 canine cells. Complete genome sequencing was conducted using Sanger method.

RESULTS Two of eight specimens contained sufficient amounts of CCoVs as confirmed by less-sensitive single-step RT-PCR assays, and one specimen demonstrated cytopathic effects (CPE) in A72 cells. Complete genome sequencing of the virus causing CPE identified it as a novel canine-feline recombinant alphacoronavirus (genotype II) that we named CCoV-HuPn-2018. Most of CCoV-HuPn-2018 genome is more closely related to a CCoV TN-449, while its S gene shared significantly higher sequence identity with CCoV-UCD-1 (S1 domain) and a feline CoV WSU 79-1683 (S2 domain). CCoV-HuPn-2018 is unique for a 36 nt (12-aa) deletion in the N protein and the presence of full-length and truncated 7b non-structural protein which may have clinical relevance.

CONCLUSIONS This is the first report of a novel canine-feline recombinant alphacoronavirus isolated from a human pneumonia patient. If confirmed as a pathogen, it may represent the eighth unique coronavirus known to cause disease in humans. Our findings underscore the public health threat of animal CoVs and a need to conduct better surveillance for them.

Keywords: canine coronavirus; novel alphacoronavirus; pneumonia; zoonotic disease; East Malaysia

Human coronaviruses (HCoVs) associated with common colds (HCoV-229E and HCoV-OC43) were initially identified in the mid-sixties, and two more, HCoV-NL63 and HCoV-HKU1, were described in 2004 and 2005, respectively [1-3]. The emergence of severe acute respiratory syndrome (SARS) coronavirus (CoV) in 2002-2003 and Middle East respiratory syndrome (MERS)-CoV in 2012 has demonstrated that CoVs can cause severe-to-fatal disease [4]. Evidence suggests that bats are likely to be the original source of SARS-CoV and MERS-CoV [5, 6]. The most recent and notable CoV-related threat is represented by the COVID-19 pandemic caused by SARS-CoV-2 [7]. While the origin of SARS-CoV-2 is still debated [8], it is thought to have emerged via a spillover event originating at a Chinese wet market. Thus, zoonotic CoVs pose a major threat to human health with different animals serving as natural reservoirs/intermediate hosts to CoVs transmittable to humans [9, 10]. However, the potential threat represented by cats and dogs or their CoVs has been sparsely studied.

Different genotypes (I,II) of canine CoVs (CCoVs) of *Alphacoronavirus 1* species cause moderate-to-severe enteric disease in dogs [11]. CCoV-II circulation has been confirmed in dogs since 1971, while CCoV-I was discovered about 3 decades later [12, 13]. TGEV, CCoV-II and feline CoV (FCoV)-II have reportedly originated from CCoV-I and FCoV-I through gene loss and recombination [14]. Similar to FCoVs, CCoV-I strains do not grow or grow poorly in cell culture and their cellular receptor is unknown, while CCoV-II strains grow readily in culture utilizing aminopeptidase N (APN) as a cellular receptor [15]. This emphasizes the complex evolution of CCoVs/*Alphacoronavirus 1* species and their ability to infect different hosts inducing variable clinical disease. It has been demonstrated recently that another CoV, porcine deltacoronavirus, utilizing APN as a cellular receptor can infect cells of an unusually broad species origin, including human and chicken [16].

Our recent studies documenting CCoV in human pneumonia patients in Sarawak [17] and FCoV-like CoVs in human patients with acute respiratory symptoms in Arkansas [18] represent the only

evidence that *Alphacoronavirus 1* species may infect and be associated with a clinical disease in humans. Here we report isolation, complete genome sequencing and molecular analysis of a CCoV virus from one of the pneumonia patients.

METHODS

SAMPLE SOURCE, SCREENING AND CELL CULTURE ISOLATION

Eight of 301 nasopharyngeal swab (NPS) specimens from hospitalized pneumonia patients (2017-18 at Sibu and Kapit Hospitals, Sarawak, Malaysia) were previously confirmed to contain CCoV using a semi-nested RT-PCR assay and Sanger sequencing (**Tables 1 and S1**)[17]. The eight pneumonia patients all came from Sibu Hospital (**Table 1**). Seven (87.5%) were less than five years in age, four were infants, and most were from Sarawak's indigenous ethnic groups who typically live in rural/suburban longhouses or villages. Seven (87.5%) of the patients had evidence of a viral co-infection (**Table 1**). All bacterial blood cultures were negative, and all patients hospitalized for 4-6 days and recovered.

RNA EXTRACTION AND RT-PCR

RNA was extracted from suspended NPS samples using the 5X MagMAX Viral Isolation Kit (Applied Biosystems). Because one-step RT-PCR is less sensitive than nested/semi-nested RT-PCR, further characterization was conducted using one-step RT-PCR assays to ensure no contamination. Qiagen One-step RT-PCR kit was used with the indicated primers and cycling protocols (**Table S2**). Amplicons generated with CCoV-N-F/CCoV-N-R primers were gel-extracted using QIAquick® Gel Extraction Kit (Qiagen) and sequenced using Sanger method at the Molecular and Cellular Imaging Center (MCIC) at The Ohio State University (OSU), Ohio Agricultural Research and Development Center (Wooster, OH).

VIRUS ISOLATION IN A72 CELL CULTURE AND TRANSMISSION ELECTRON MICROSCOPY

Canine fibroblast tumor (A72) cells (received from Dr. Alfonso Torres, Cornell College of Veterinary Medicine) were maintained and used for sample inoculation as described previously [19]. Serially diluted NPS fluids (1:10-1:10,000) were used to inoculate the A72 monolayers. After 72 hours the infected cells and medium were harvested and used for RNA extraction with the RNEasy Mini Kit (Qiagen). The immune transmission electron microscopy (I-TEM) was conducted as described previously using polyclonal anti-canine coronavirus guinea pig serum (BEI Resources, NR-2727), the I-TEM images were captured at the MCIC [20].

COMPLETE GENOME SEQUENCING USING THE SANGER METHOD

The viral RNA was converted into cDNA using a SuperScript III cDNA synthesis kit (Invitrogen, USA).

Forty-two primer pairs (**Table S3**) covering the whole genome were designed based on the sequence of CCoV, strain TN-449, the most closely related strain as determined by BLASTn analysis of the partial N gene sequence of the newly identified CCoV for which the complete genome was available. Using these primers and Taq Platinum (Invitrogen) 12 amplicons (1.7-3.6 kb) were generated and purified using the QIAquick® Gel Extraction Kit and sequenced with 3× coverage using the Sanger dideoxy method with a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, USA) at the MCIC and at the James Comprehensive Cancer Center Shared Genomics Core, The Ohio State University (Columbus, OH). After the initial analysis/sequence assembly, seven additional primer pairs were designed based on the newly generated sequences to close the remaining gaps (**Table S3**). The fragments were amplified and sequenced as described

above. The 5'- and 3'-genomic ends were amplified using the 5' and 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's manual.

SEQUENCE ASSEMBLY AND ANALYSIS

Raw sequences were trimmed to remove low-quality reads and amplicon-primer linkers. Each ORF was analyzed using Viral Genome ORF Reader (VIGOR) to predict viral protein sequences. The annotated CCoV genome was submitted to the GenBank (accession number is MW591993). The alignments were further analyzed Sequence Manipulation Suite (SMS, Version 2) (<https://www.bioinformatics.org/sms2/>) to determine nt identities between the reference and newly generated sequences. Sequence alignment and phylogenetic analysis were performed using the ClustalW method and the maximum-likelihood method with the general time reversible nucleotide substitution model and bootstrap tests of 1,000 replicates of MEGAX software. The CoV genomes for reference strains from GenBank used in the phylogenetic analyses are listed in (Table 3). The RIP (Recombinant Identification Program; <http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html>) was used to identify recombination points within the CCoV-HuPn-2018 genome with the following parameters: window size of 400 and confidence threshold of 90%. Glycosylation prediction was conducted using the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

RESULTS

RT-PCR AND PARTIAL SEQUENCING OF CCoV

Samples from two of the eight patients from whom CCoV was earlier detected were positive in universal and CCoV-specific one-step RT-PCR assays (Table S2). This could be due to different quantity/integrity of CCoV in samples collected at variable time-points post infection. According to the BLASTn search, the sequences obtained for both samples using CCoV-N-F/CCoV-N-R primers

shared the highest nucleotide (nt) identity (96.31%) with several CCoV strains including TN-449 and HLJ-073 (listed in **Table 3**). We selected TN-449 sequence to design sequencing primers covering the complete genome (**Table S3**).

CCoV REPLICATION IN A72 CANINE CELLS

While eight CCoV-positive NPS samples were inoculated into A72 cells, only one sample (1153, **Table 1**) produced CPE in the cells (**Figure S1**). The A72 cell-passaged material (P1) was inoculated into A72 cells again and CPE was observed within the same timeframe (P2). RNA extracted from both P1 and P2 tested CCoV positive; RNA extracted from P1 was used for complete genome sequencing. This virus was visualized using I-TEM (**Figure 1**) and is referred to as *CCoV-HuPn-2018* throughout (HuPn - human pneumonia).

GENOMIC ORGANIZATION OF CCoV-HuPn-2018

The assembled viral genome was 29,083/29,351 (due to differences between the two 7b forms) nt long excluding the poly(A) tail. The genomic organization and gene order were typical of that of other *Alphacoronavirus 1* species: ORF1a1b, spike (S), ORF3a, ORF3b, ORF3c, envelope (E), membrane (M), nucleocapsid (N), ORF7a and ORF7b (**Figure S2, Table 2**). The structural and non-structural proteins (NSPs) were flanked by 5'- and 3'-untranslated regions with 3'- poly(A) tail.

The 5'-UTR consisted of 313 nt including the leader sequence (nt1–94) and the conserved core 5-CU(T)AAAC-3 (nt95–100) of the transcription regulatory sequence (TRS) that controls the mRNA synthesis during the subgenomic RNA discontinuous transcription. Similar TRS signals preceded five genes: S (nt 20,335), 3a (nt 24,787), E (nt 25,866); M (nt 26,156); N (nt 26,951); and 7a/b (nt 28,072) (**Table 2**). There were no TRS signals in front of 3b/3c and 7b suggesting that they may be expressed from polycistronic mRNAs. The 3'-end of the viral genome consists of a 275-nt 3'-UTR followed by the poly(A) tail.

Twenty thousand sixty-one nt following the 5'-UTR were occupied by the replicase gene encoding for two large polyproteins (pp), pp1a and pp1b, with pp1ab being synthesized through ribosomal slippage at position 12,339 as reported for highly related CCoV TN-449.

The SMS analysis demonstrated that the genome was mostly similar to CCoV strains TN-449, HLJ-073 and A76 and transmissible gastroenteritis virus (TGEV) Purdue strain sharing 93.31%, 91.744%, 90.63% and 91.47% nt identity, followed by feline CoV (FCoV)/feline infectious peritonitis virus (FIPV) strains (83.96-84.58% nt identity) (**Table 3**). This suggests CCoV-HuPn-2018 represents a novel strain within the *Alphacoronavirus 1* species.

Similar to the complete genome, CCoV-HuPn-2018 ORF1ab region shared the highest nt identity with that of TN-449 (95.84%), HLJ-073 (95.70%) and A76 (95.40%), followed by other CCoV (89-94.28%), various TGEV (92.6-94.49%) and FCoV (82.08-85.84%) strains.

Further, while the full-length S gene of CCoV-HuPn-2018 shared the highest nt identity with CCoV TN-449 (93.42%), its S1 domain was near identical to that of CCoV UCD-1 (for which only S1 sequence is available) sharing 99.19% nt identity, which was higher than for any other genomic region (**Table 3**). The S2 domain of CCoV-HuPn-2018 shared the highest identity of 97.13% with FCoV WSU 79-1683, providing additional evidence of recombinant (feline-canine, canine-TGEV) nature of most CCoV S genes.[21]

The remaining three genes encoding for structural proteins E, M and N shared the highest nt identity of 95.18%, 97.08% and 93.77%, respectively, with those of CCoV A76 (**Table 3**).

PHYLOGENETIC ANALYSIS

Phylogenetic analysis of complete genome sequences demonstrated that the novel CCoV-HuPn-2018 formed a monophyletic branch with CCoV, TGEV, FCoV strains and swine enteric CoV [TGEV with porcine epidemic diarrhea virus (PEDV) recombinant S gene]

(**Figure 2A**). Further, the full-length S gene of the CCoV-HuPn-2018 was closely related to CCoV strains and TGEV Purdue (**Figure 2B**), while its S1 and S2 domains were most closely related to CCoV UCD-1 and FCoV WSU 79-1683, respectively (**Figure 2C and 2D**).

Phylogenetic analysis of the E gene confirmed the close relatedness between CCoV-HuPn-2018 and CCoV A76; however, due to the high level of conservation of this gene, all of the analyzed *Alphacoronavirus 1* strains with the exception of FIPV 79-1146 formed a tight cluster (**Figure 2E**). The M and N gene phylogenetic analysis confirmed that they were highly similar between CCoV-HuPn-2018 and CCoV A76, followed by other CCoVs and TGEV, while FCoVs formed separate clusters supporting higher degree of divergence in this genomic region evident from SMS analysis (**Figure 2F and 2G; Table 3**).

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RECOMBINATION ANALYSIS

Potential recombination breakpoints between the background CCoV and TGEV strains were present throughout the ORF1ab, resulting in the short regions sharing more similarity with HLJ-073, A76 and TGEV Purdue strain (**Figure 3A**). Additionally, while the first two-thirds of the ORF1ab was relatively dissimilar between the CCoV-HuPn-2018 and FCoV WSU 79-1683/FIPV 79-1146, the similarity was higher (and comparable to CCoV/TGEV strains) in the last third with multiple recombination breakpoints (**Figure 3A**). The 3'-end of the genome downstream from the S gene was most similar between CCoV-HuPn-2018 and CCoV strain A76. While the S2 domain shared the highest similarity with that of FCoV WSU 79-1683, the sequence similarity between the CCoV-HuPn-2018 and all the background sequences in the hypervariable S1 region was low. Thus, this finding is consistent with the SMS and phylogenetic analysis results and indicates the recombinant nature of this strain (**Figure 3A**).

The S gene RIP analysis revealed the presence of the recombination point at ~2kb, with the S2 domain being highly similar to FCoV WSU 79-1683 as noted above (**Figure 3B and 3C**). The S1 domain RIP analysis allowed us to include the CCoV UCD-1 S1 domain into the analysis and confirm that it indeed shared the highest similarity with the CCoV-HuPn-2018 S1. These observations confirmed that the novel strain carries a recombinant CCoV/FCoV S protein.

1 STRUCTURAL/NON-STRUCTURAL PROTEIN ANALYSIS

2
3 The S protein was comprised of 1,448 aa similar to other CCoV II strains and shorter than S proteins
4 of CCoV I characterized previously [22]. Twenty-nine potential glycosylation sites were predicted in the
5 S protein of the newly identified CCoV-HuPn-2018 (**Figure S3A**) similar to other CCoVs.[22] Unlike
6 CCoV I, some FCoV and all beta- and gammacoronaviruses, the characteristic multibasic motif (RRXRR)
7 – furin recognition site was absent in the S protein of CCoV-HuPn-2018, suggesting that the virus carries
8 an uncleaved S protein, similar to most other alphacoronaviruses [15]. Thus, this novel strain shares more
9 similarities with CCoV-II strains.

10 Surprisingly, there were no unique deletions or insertions in the S protein of CCoV-HuPn-2018. Also,
11 there were a total of 5 aa differences between the CCoV-HuPn-2018 and CCoV UCD-1 in the S1 domain;
12 however, these aa were identical to those found in the TGEV Purdue S1 and not unique.

13 The E protein was 81-aa long and did not contain any N-glycosylation sites, whereas three N-
14 glycosylated residues have been predicted in each the 261-aa long M and the 370-aa long N proteins
15 (**Figures S3B and S3C**), which is similar to several other FCoV/CCoV strains. While no evidence of
16 recombination was observed for E, M or N proteins, the N protein contained a unique 12-aa deletion
17 within the SR-rich region (located between aa 164 and 177 for other CCoV strains). Presence of this
18 deletion was confirmed in the original NSP samples 1116 and 1153.

19 The three ORFs 3a, 3b and 3c between S and E genes encoded for proteins with sizes of 71, 71 and
20 244 aa, respectively. ORF3, previously found in CCoV I genomes only [14, 22], was not present in the
21 new strain. The 3'-end accessory protein gene 7a encoded for 101-aa, while there were at least 2 forms of
22 7b: full-length (213-aa) and the one with a 227-nt deletion (leading to a frame shift and premature
23 truncation of the putative protein).

24 DISCUSSION

26 Our previous study identified eight pneumonia patients with molecular evidence of CCoV in their
27 NPS specimens [17]. Partial sequencing and BLASTn analysis suggested that these were closely related
28 but distinct CCoV variants (**Table S1**). The eight pneumonia patients were mainly children living in long
29 houses or villages in rural/suburban areas where domestic animal and jungle wildlife exposure with the
30 family is common.

31 Here, we confirmed the presence of CCoV with different, less sensitive, one-step RT-PCR assays in
32 two specimens, grew a virus in A72 cells from one specimen, and conducted a complete genome
33 sequence analysis of the CCoV. Our results demonstrated that CCoV-HuPn-2018 is a novel canine-feline-
34 like recombinant strain with a unique N. To our knowledge, this is the first report suggesting that a CCoV
35 without major genomic re-arrangements or adaptive modifications in the S protein might replicate in
36 association with pneumonia in a human host.

37 The conducted analyses demonstrated that the newly identified CCoV-HuPn-2018 was most closely
38 related to CCoV TN-449, while its S1 and S2 domains shared the highest nt identity with that of CCoV
39 UCD-1 and FCoV WSU 79-1683, respectively. These findings are suggestive of the recombinant nature
40 of this strain, similar to many previously characterized CCoVs [21]. Phylogenetic and recombinational
41 analyses confirmed that CCoV-HuPn-2018 was only distantly related to other alphacoronavirus species
42 including HCoVs (229E and NL63) and bat CoVs and likely originated via multiple recombination events
43 between different *Alphacoronavirus 1* strains, but not other alphacoronaviruses.

44 The ability of the novel strain to replicate in A72 canine cells, the absence of ORF3, the higher
45 overall similarity with CCoV-II strains (TN-449 and HLJ-073) and the lack of the furin cleavage site
46 between S1 and S2 domains suggest that it belongs to CCoV genotype II [22].

47 The unique feature not found in any other known CCoVs and *Alphacoronavirus 1* species, namely the
48 12-aa deletion in the middle portion of the N protein was confirmed in both original NSP samples 1153
49 and 1116. While insertions/deletions in the N protein are not found among the known *Alphacoronavirus 1*

50 strains, the deletion of the SR-rich domain within the middle region of SARS-CoV N protein reportedly
51 resulted in dramatic changes in its cellular localization soon after its zoonotic transmission [23]. Thus,
52 similar to SARS-CoV, CCoV-HuPn-2018 possesses some unique genetic features suggestive of recent
53 zoonotic transmission. Notably, such N protein re-arrangements are characteristic for SARS-CoV/SARS-
54 CoV-2 with higher case-fatality rates [24].

55 While SARS-CoV and FCoV NSP7b was not essential for viral replication *in vitro* and *in vivo*
56 experiments, its deletion/truncation may be associated with attenuated phenotype [25]. Disruption in the
57 expression of the NSPs following zoonotic transmission of SARS-CoV was reported previously
58 suggesting it may represent an adaptive mechanism [26]. Finally, deletions unique to FIPVs were found
59 in ORFs 3c and/or 7b that were hypothesized to be responsible to the shift from enteric (FCoV) to FIPV
60 phenotype and increased pathogenicity [27].

61 The ability of CCoV to quickly evolve via frequent recombination events and induce disease of
62 variable severity is even more concerning given these data indicating that circulating CCoV may already
63 be transmittable to humans.

64 This study had a number of limitations. First, we have not met recognized standards of causality such
65 as Koch's postulates or Bradford Hill criteria. Second, we recognize that the detected CCoVs could only
66 be "carried" in some of the eight patients' airways not causing disease. However, identification of: 1)
67 FCoV-like CoVs in influenza-negative patients with acute respiratory symptoms in Arkansas and 2)
68 porcine deltacoronavirus in children in Haiti further emphasizes that *Alphacoronavirus 1* species may be
69 infectious/pathogenic to humans [18, 28].

70 In conclusion, we recovered and characterized a novel recombinant coronavirus, CCoV-HuPn-2018,
71 from a hospitalized pneumonia patient. While possessing some unique characteristics likely suggestive of
72 a recent zoonotic transmission, this novel strain with recombinant CCoV UCD-1/FCoV WSU 79-1683 S
73 protein shares multiple genomic features of wide-spread CCoV-II. Further studies to investigate CCoV

74 prevalence, seroprevalence and pathogenic potential in humans are needed. Additional studies should be
75 conducted to evaluate the biological relevance of the observed deletion in the N protein.

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NOTES

AUTHOR CONTRIBUTIONS

ANV – designed, overseen and provided financial support for the experiments on CCoV-HuPn-2018 characterization and sequences, sequenced parts of the genome, analyzed the data and wrote the manuscript; AD – conducted most of the experiments on CCoV-HuPn-2018 cell culture isolation and Sanger sequencing; DD - conducted some of the experiments on Sanger sequencing; LX – screened 301 samples and identified the eight samples positive for CoV/CCoV; THT – coordinated sample collection, obtaining ethical clearance, collection and processing of the patient demographic data; JSYL - coordinated sample collection, obtaining ethical clearance, collection and processing of the patient demographic data; LJS – critically revised the manuscript draft; GCG – led the original studies, oversaw this new study, provided financial support and revised the manuscript.

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ethical standards of the Malaysian Ministry of Health's Medical Research and Ethics Committee (protocol number NMRR-17-316-34395), the Duke University Health System Institutional Review Board, DukeNUS Medical School Ethical Review Board, and the Naval Medical Research Center-Asia Human Research Protection Program (HRPO no. W911QY-16-D-0058).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. Demographic and clinical characteristics of the eight patients with molecular evidence of canine coronavirus in their nasopharyngeal swab specimen.

ID	Gender /Age/Ethnicity	Town / Housing Type (Number of cohabitants at home)	Underlying condition /medication	Known exposure to animals	Highest oxygen support during admission	Duration of hospital stay (complete days)	Other concomitant pathogens detected*
1090	Male /13½ months/Iban	Sibu/unknown (2)	Pre-school wheeze / inhaled Budesonide	No	No information	No information	Adenovirus
1116	Male/9½ months/Iban	Sibu/longhouse (9)	Glucose-6-phosphate dehydrogenase deficiency/oral amoxicillin	No	Nasal prong oxygen 1 L/min	5	Adenovirus
1126	Female/2½ years/Iban	Bintulu/longhouse (9)	None	No	No information	No information	Parainfluenza virus 3
1128	Female/11 months/Iban	Sibu/longhouse (7)	None	Cats	Nasal prong oxygen 1L/min	5	Parainfluenza virus 3
1131	Female/4¼ years/Chinese	Sibu/townhouse (4)	None	Cats and dogs	No information	No information	Influenza A
1153	Male/5½ months/Melau	Daro/village (10)	None/oral ampicillin and cloxacillin	No	No information	No information	Rhinovirus C
1157	Female/10 months/Bidayuh	Julau/longhouse (4)	Pre-school wheeze/inhaled fluticasone	No	Nasal prong oxygen 1L/min	6	Adenovirus
2062	Female/37½ years/Iban	Sibu/staff quarter (15)	Bronchial asthma/inhaled Fluticasone	Dogs	Nasal prong oxygen 3L/min	4	None

CCoV-HuPn-2018 was isolated from sample 1153, bolded. *The patients' nasopharyngeal swabs were studied with molecular assays for adenovirus, human enterovirus, influenza A, B, C, and D, respiratory syncytial virus A and B, parainfluenza viruses 1, 2, 3 and 4, and rhinovirus [29-31].

Table 2. Complete genome and individual gene length and other characteristics of CCoV-HuPn-2018

Genomic region/ORF number	Coding sequence	Length, nt	Putative TRS start nt/sequence		Protein name	Protein size, aa	Note
			Start nt position	Sequence			
5'-UTR	N/A	313	N/A	N/A	N/A	N/A	Similar to other CCoVs
3'-UTR	N/A	275	N/A	N/A	N/A	N/A	
ORF1/b	314-20,374	20,061	90	TCGAACTAAACGAAAT	Pp1ab	6,686	
ORF2	20,371-24,717	4,347	20,335	GTTACTAAACTTTG	S	1,448	Recombinant structure with the S1 domain most closely related to CCoV UCD-1 and the S2 domain most closely related to FCoV WSU 79-1683
ORF3a	24,820-25,035	216	24,787	AGAACTAAACTTATG	3a	71	Only one TRS before 3a was found; so 3a, 3b and 3c are likely to be expressed from polycistronic mRNAs
ORF3b	24,980-25,195	216	N/A	N/A	3b	71	
ORF3c	25,192-25,926	735	N/A	N/A	3c	244	
ORF4	25,913-26,158	246	25,866	GGTTCTAAACGAAAT	E	81	No unique features
ORF5	26,169-26,954	786	26,156	TGAACTAAACAAAAT	M	261	
ORF6	26,967-28,079	1,113	26,951	ATAACTAAACTTCTA	N	370	Contains 36 nt deletion in the middle region
ORF7a	28,084-28,389	306	28,072	CGAACTAAACGAATG	7a	101	Only one TRS before 7a is located; 7b is being expressed from polycistronic mRNAs
ORF7b*	28,394-28,808/29,035	415/642	N/A	N/A	7b	34/213	*Truncated, likely non-functional, contains an out of frame 227-nt deletion close to its 5' end followed by premature stop codons, and full-length forms.

Table 3. Percent (%) identities of CCoV-HuPn-2018 to Alphacoronavirus-1 reference strains in the complete genomic sequence and genes for structural proteins.

<i>Alphacoronavirus 1</i>	Strain	Accession number	Nt Identity (%) to CCoV-HuPn-2018						
			Complete genome	S	S1	S2	E	M	N
CCoV-IIa	TN-449	JQ404410.1	93.31%*	93.42%	73.22%	95.20%	93.57%	95.08%	93.42%
CCoV-IIa	HLJ-073	KY063618.2	91.74%	93.33%	73.32%	95.20 %	93.17%	95.08%	93.33%
CCoV-IIc	A76	JN856008.2	90.63%	93.77%	53.80%	85.42%	95.18%	97.08%	93.77%
CCoV	UCD-1	AF116248.1	N/A	N/A	99.19%	N/A	N/A	N/A	N/A
TGEV	Purdue (virulent)	DQ811789.2	91.47%	92.12%	90.93%	94.59%	93.98%	92.65%	92.12%
FCoV-II	WSU 79-1683	JN634064.1	84.58%	74.91%	72.80%	97.13%	93.68%	86.25%	74.91%
FCoV-II/FIPV	79-1146	DQ010921.1	84.04%	75.5%	73.04%	95.04%	79.92%	81.77%	75.5%

* Blue shading indicates the highest nt identity between CCoV-HuPn-2018 and a given strain.

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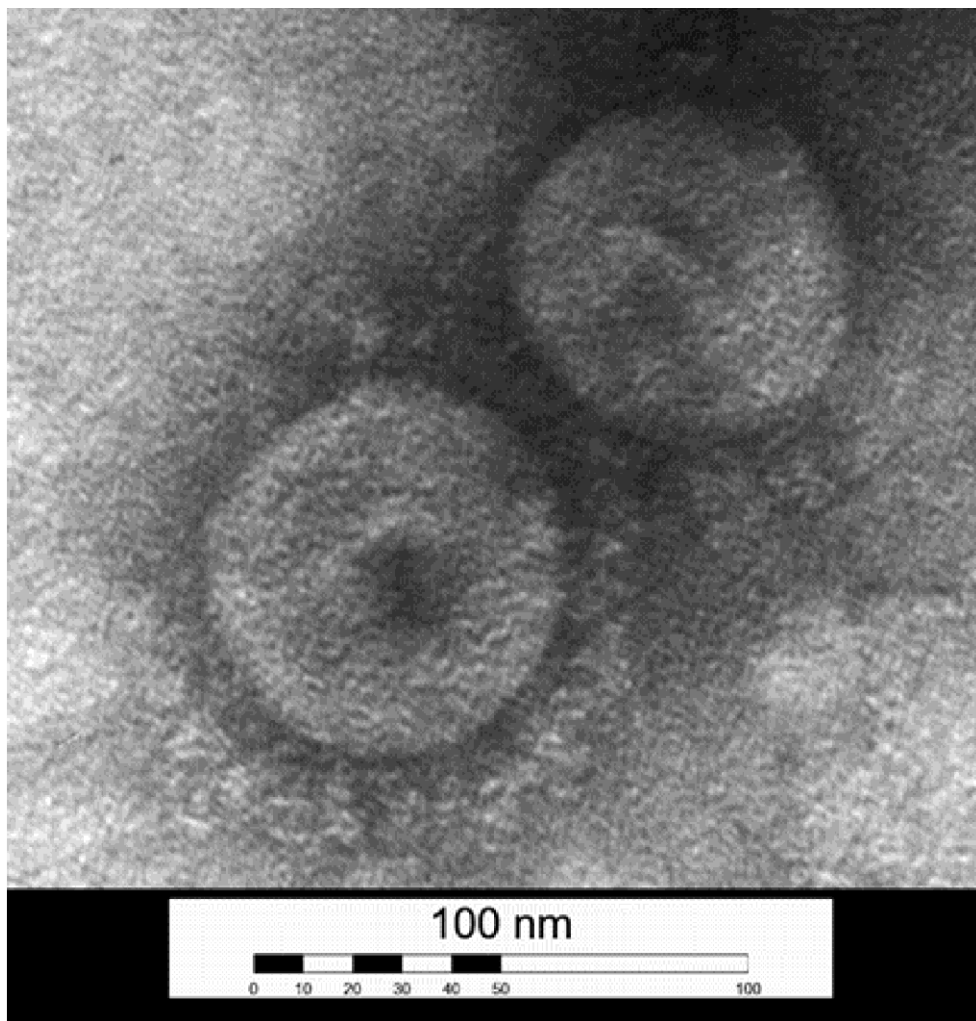
FIGURE LEGENDS

Figure 1. Immune transmission electron microscopy image of CCoV-HuPn-2018 from an A72 cell culture. The sample was incubated with anti-canine coronavirus guinea pig serum, leading to the specific viral-antibody aggregates. The bar represents 100 nm.

Figure 2. Phylogenetic tree based on complete genome (A), S gene (B), S1 (C), S2 domain (D), E gene (E), M gene (F) and N gene (G) sequences of the CCoV-HuPn-2018 viral isolate and other alphacoronavirus species. Bootstrap values are represented at key nodes. Scale bar indicates nucleotide substitutions per site. CCoV, canine coronavirus; TGEV, transmissible gastroenteritis virus; FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; HCoV, human coronavirus; SADS-CoV, swine acute diarrhea syndrome coronavirus; BtCoV, bat coronavirus; PEDV, porcine epidemic diarrhea virus. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. This analysis involved 13 nucleotide sequences. Evolutionary analyses were conducted in MEGA X. The newly identified viral isolate CCoV-HuPn-2018 labeled with black circle marker.

Figure 3. Recombinational analysis of the CCoV-HuPn-2018 complete genome (A), S1 (B) and S2 (C) domains. At each position of the window, the query sequence CCoV-HuPn-2018 was compared with background sequences for six strains shown in the legend on the right. The x-axis represents the length of the genome, and the y-axis represents the similarity value. When the query sequence is similar to the background sequence(s), the homologous regions are indicated as thick dashed lines (of the corresponding color) on the top of the plot. Arrows represent potential recombination breakpoints.

Figure 1



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Figure 2

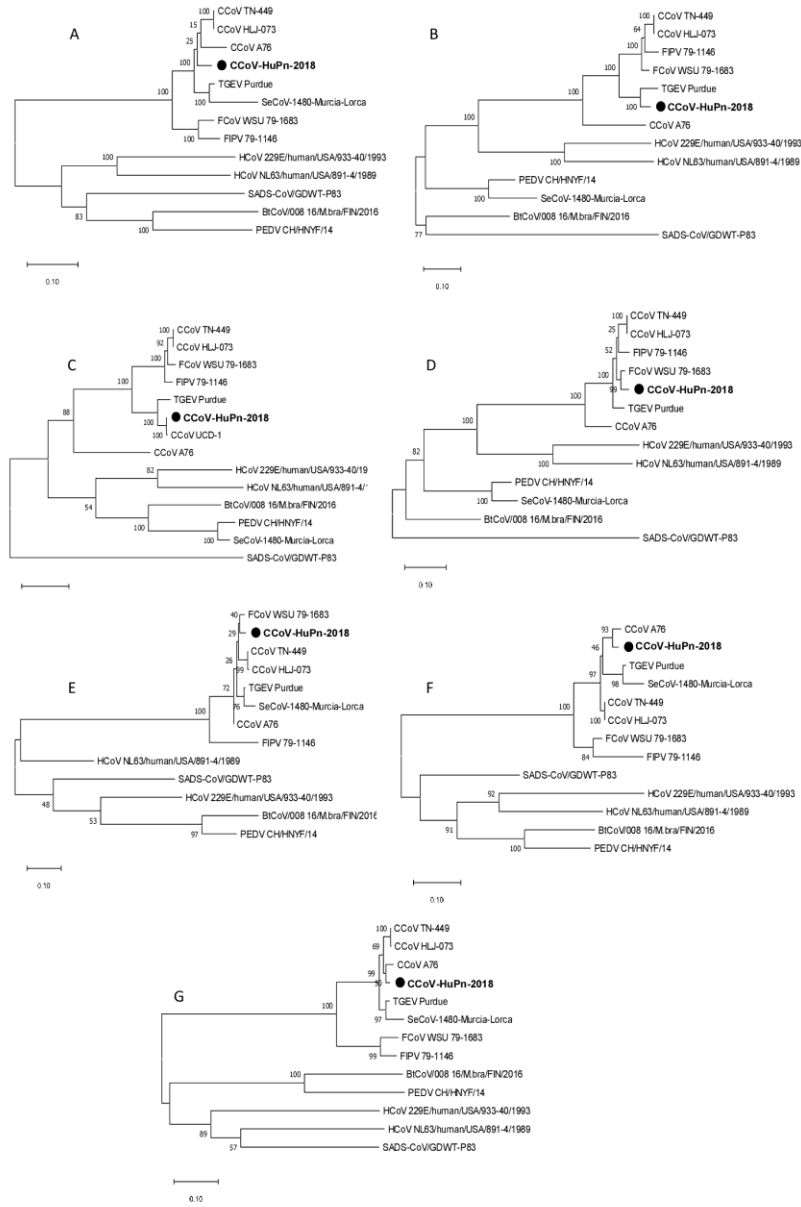


Figure 3

