Clinical Infectious Diseases
MAJOR ARTICLE

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Novel Canine Coronavirus Isolated from a Hospitalized Patient With Pneumonia in East Malaysia

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Background. During the validation of a highly sensitive panspecies coronavirus (CoV) seminested reverse-transcription polymerase chain reaction (RT-PCR) assay, we found canine CoV (CCoV) RNA in nasopharyngeal swab samples from 8 of 301 patients (2.5%) hospitalized with pneumonia during 2017–2018 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 1-step RT-PCR assays, and viral isolation was performed in A72 canine cells. Complete genome sequencing was conducted using the Sanger method.

Results. Two of 8 specimens contained sufficient amounts of CCoVs as confirmed by less-sensitive single-step RT-PCR assays, and 1 specimen demonstrated cytopathic effects in A72 cells. Complete genome sequencing of the virus causing cytopathic effects identified it as a novel canine-feline recombinant alphacoronavirus (genotype II) that we named CCoV–human pneumonia (HuPn)–2018. Most of the 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-nucleotide (12–amino acid) deletion in the N protein and the presence of full-length and truncated 7b nonstructural protein, which may have clinical relevance.

Conclusions. This is the first report of a novel canine-feline recombinant alphacoronavirus isolated from a human patient with pneumonia. 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-1960s, and 2 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 CoV in 2012 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 Middle East respiratory syndrome CoV [5, 6]. The most recent and notable CoV-related threat is represented by the coronavirus disease 2019 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 or 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, and CCoV-I was discovered about 3 decades later [12, 13]. Transmissible gastroenteritis virus (TGEV), CCoV-II, and feline CoV (FCoV) II have reportedly originated from CCoV-I and FCoV-I through gene loss and recombination [14]. Similarly 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 using aminopeptidase N 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

Received 5 March 2021; editorial decision 10 May 2021; published online 20 May 2021.
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Clinical Infectious Diseases® 2022;74(3):446–54
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DOI: 10.1093/cid/ciab456

been demonstrated that another CoV, using aminopeptidase N as a cellular receptor, porcine deltacoronavirus, can infect cells of unusually broad species origin, including human and chicken [16].

Previous studies documenting CCoV in human patients with pneumonia 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 patients with pneumonia.

METHODS

Sample Source, Screening, and Cell Culture Isolation

Eight of 301 nasopharyngeal swab (NPS) specimens from hospitalized patients with pneumonia (2017–2018 at Sibu and Kapit Hospitals, Sarawak, Malaysia) were previously confirmed to contain CCoV using a seminested reverse-transcription polymerase chain reaction (RT-PCR) assay and Sanger sequencing (Table 1 and Supplementary Table 1) [17]. The 8 patients with pneumonia all came from Sibu Hospital (Table 1). Seven (87.5%) were aged <5 years, 4 were infants, and most were from Sarawak's indigenous ethnic groups, who typically live in rural or suburban longhouses or villages. Seven of the patients (87.5%) had evidence of a viral coinfection (Table 1). All bacterial blood cultures were negative, and all patients were 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 1-step RT-PCR is less sensitive than nested or seminested RT-PCR, further characterization was conducted using 1-step RT-PCR assays to ensure no contamination. A Qiagen 1-step RT-PCR kit was used (primers and cycling protocols provided in Supplementary Table 2). Amplicons generated with CCoV-N-F/CCoV-N-R primers were gel extracted using the QIAquick Gel Extraction Kit (Qiagen) and sequenced using the Sanger method at the Molecular and Cellular Imaging Center (MCIC) at the Ohio Agricultural Research and Development Center, The Ohio State University, Wooster.

Virus Isolation in A72 Cell Culture and Transmission Electron Microscopy

Canine fibroblast tumor (A72) cells (received from Alfonso Torres, Cornell College of Veterinary Medicine) were maintained and used for sample inoculation, as described elsewhere [22]. 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). Immune transmission electron microscopy (I-TEM) was conducted as described elsewhere,

Table 1. Demographic and Clinical Characteristics of 8 Patients With Molecular Evidence of Canine Coronavirus in Nasopharyngeal Swab Specimens

ID	Sex/Age/Ethnicity	Town/Housing Type (No. of Cohabitants at Home)	Underlying Condition/Medication	Known Exposure to Animals	Highest Oxygen Support During Admission	Duration of Hospital Stay	Other Concomitant Pathogens Detected[a]
1090	Male/13.5 m/Iban	Sibu/unknown (2)	Preschool wheeze/inhaled budesonide	No	No information	No information	Adenovirus
1116	Male/9.5 m/Iban	Sibu/longhouse (9)	Glucose-6-phosphate dehydrogenase deficiency/oral amoxycillin	No	Nasal prong oxygen (1 L/min)	5 Complete days	Adenovirus
1126	Female/2.5 y/Iban	Bintulu/longhouse (9)	None	No	No information	No information	Parainfluenza virus 3
1128	Female/11 m/Iban	Sibu/longhouse (7)	None	Yes (cats)	Nasal prong oxygen (1 L/min)	5 Complete days	Parainfluenza virus 3
1131	Female/4.5 y/Chinese	Sibu/townhouse (4)	None	Yes (cats and dogs)	No information	No information	Influenza A
1153[b]	Male/5.5 m/Melanau	Daro/village (10)	None/oral ampicillin and cloxacillin	No	No information	No information	Rhinovirus C
1157	Female/10 m/Bidayuh	Julau/longhouse (4)	Preschool wheeze/inhaled fluticasone	No	Nasal prong oxygen (1 L/min)	6 Complete days	Adenovirus
2062	Female/37.5 y/Iban	Sibu/staff quarter (15)	Bronchial asthma/inhaled fluticasone	Yes (dogs)	Nasal prong oxygen (3 L/min)	4 Complete days	None

Abbreviation: ID, patient identifier.

[a] Patients' nasopharyngeal swab specimens 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 [19–21].

[b] Canine coronavirus–human pneumonia–2018 was isolated from sample 1153.

using polyclonal anti-CCoV guinea pig serum (BEI Resources; NR-2727); the I-TEM images were captured at the MCIC [23].

Complete Genome Sequencing With the Sanger Method

The viral RNA was converted into complementary DNA (cDNA) using a SuperScript III cDNA synthesis kit (Invitrogen). Forty-two primer pairs (Supplementary Table 3) covering the whole genome were designed based on the sequence of CCoV, strain TN-449, the most closely related strain, as determined by The Basic Local Alignment Search Tool (BLAST) nucleotide (BLASTn) analysis of the partial N gene sequence of the newly identified CCoV for which the complete genome was available. Using these primers and Platinum Taq (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), at the MCIC and at the James Comprehensive Cancer Center Shared Genomics Core, The Ohio State University, Columbus. After the initial analysis/sequence assembly, 7 additional primer pairs were designed, based on the newly generated sequences, to close the remaining gaps (Supplementary Table 3). 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 instructions.

Sequence Assembly and Analysis

Raw sequences were trimmed to remove low-quality reads and amplicon-primer linkers. Each open reading frame (ORF) was analyzed using Viral Genome ORF Reader (VIGOR4) to predict viral protein sequences. The annotated CCoV genome was submitted to GenBank (accession no. MW591993). The alignments were further analyzed using the Sequence Manipulation Suite (SMS; version 2) (https://www.bioinformatics.org/sms2/) to determine nucleotide 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 1000 replicates with MEGA X software. The CoV genomes for reference strains from GenBank used in the phylogenetic analyses are listed in (Table 2). The Recombinant Identification Program (RIP; http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html) was used to identify recombination points within the CCoV–human pneumonia (HuPn)–2018 genome, with a window size of 400 and a 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 2 of the 8 patients from whom CCoV was earlier detected were positive in universal and CCoV-specific 1-step RT-PCR assays (Supplementary Table 2). This result could be due to differences in the quantity or integrity of CCoV in samples collected at variable time points after infection. According to the BLASTn search, the sequences obtained for both samples using CCoV-N-F/CCoV-N-R primers shared the highest nucleotide identity (96.31%) with several CCoV strains, including TN-449 and HLJ-073 (listed in Table 2). We selected the TN-449 sequence to design sequencing primers covering the complete genome (Supplementary Table 3).

CCoV Replication in A72 Canine Cells

While 8 CCoV-positive NPS samples were inoculated into A72 cells, only 1 sample (sample 1153; Table 1) produced cytopathic effects in the cells (Supplementary Figure 1). The A72 cell–passaged material (P1) was inoculated into A72 cells again, and cytopathic effects were observed within the same time frame (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.

Genomic Organization of CCoV-HuPn-2018

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

The 5' UTR consisted of 313 nucleotides, including the leader sequence (nucleotides 1–94) and the conserved core 5-CU(T)AAAC-3 (nucleotides 95–100) of the transcription regulatory sequence (TRS) that controls the messenger RNA synthesis during the subgenomic RNA discontinuous transcription. Similar TRS signals preceded 5 genes: S (nucleotide 20 335), 3a (24 787), E (25 866); M (26 156), N (26 951), and 7a/b (28 072) (Table 3). There were no TRS signals in front of 3b/3c and 7b, suggesting that they may be expressed from polycistronic messenger RNAs. The 3' end of the viral genome consists of a 275-nucleotide 3' UTR, followed by the poly(A) tail. The 20 061 nucleotides following the 5' UTR were occupied by the replicase gene encoding for 2 large polyproteins, polyproteins 1a and 1b, with polyprotein 1ab synthesized through ribosomal slippage at position 12 33, as reported for the 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 the TGEV Purdue strain, sharing 93.31%, 91.744%, 90.63% and 91.47% nucleotide identity, respectively, followed by FCoV/feline infectious peritonitis virus (FIPV) strains (83.96%–84.58% nucleotide identity) (Table 2). This suggests

Table 2. Identity Between Canine Coronavirus–Human Pneumonia–2018 and *Alphacoronavirus 1* Reference Strains for Complete Genomic Sequence and Genes for Structural Proteins

			Nucleotide Identity to CCoV-HuPn-2018, %						
Alphacoronavirus 1	Strain	Accession No.	Complete Genome	S	S1	S2	E	M	N
CCoV-IIa	TN-449	JQ404410.1	93.31[a]	93.42[a]	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[a]	97.08[a]	93.77[a]
CCoV	UCD-1	AF116248.1	NA	NA	99.19[a]	NA	NA	NA	NA
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[a]	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

Abbreviations: CCoV, canine coronavirus; CCoV-HuPn-2018, CCoV–human pneumonia 2018; E, envelope; FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; HuPN, human pneumonia; M, membrane; N, nucleocapsid; NA, not available; S, spike; TGEV, transmissible gastroenteritis virus.
[a]Highest nucleotide identity between CCoV-HuPn-2018 and given strain.

that 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 nucleotide identity with those 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. Furthermore, while the full-length S gene of CCoV-HuPn-2018 shared the highest nucleotide identity with CCoV TN-449 (93.42%), its S1 domain was nearly identical to that of CCoV UCD-1 (for which only the S1 sequence is available), sharing 99.19% nucleotide identity, higher than for any other genomic region (Table 2). The S2 domain of CCoV-HuPn-2018 shared the highest identity (97.13%) with FCoV WSU 79-1683, providing additional evidence of the recombinant (feline-canine, canine-TGEV) nature of most CCoV S genes [24]. The remaining 3 genes, encoding for structural proteins E, M, and N, shared the highest nucleotide identities (95.18%, 97.08%, and 93.77%), respectively, with CCoV A76 (Table 2).

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

Recombination Analysis

Potential recombination break points 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 the TGEV Purdue strain (Figure 3A). In addition, 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 greater (and comparable to that in CCoV/TGEV strains) in the last third, with multiple recombination break points (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 approximately 2 kb, 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 in 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.

Structural/Nonstructural Protein Analysis

The S protein comprised 1448 amino acids, similar to other CCoV II strains and shorter than S proteins of CCoV I characterized elsewhere [25]. Twenty-nine potential glycosylation sites were predicted in the S protein of the newly identified CCoV-HuPn-2018 (Supplementary Figure 3*A*), similar to

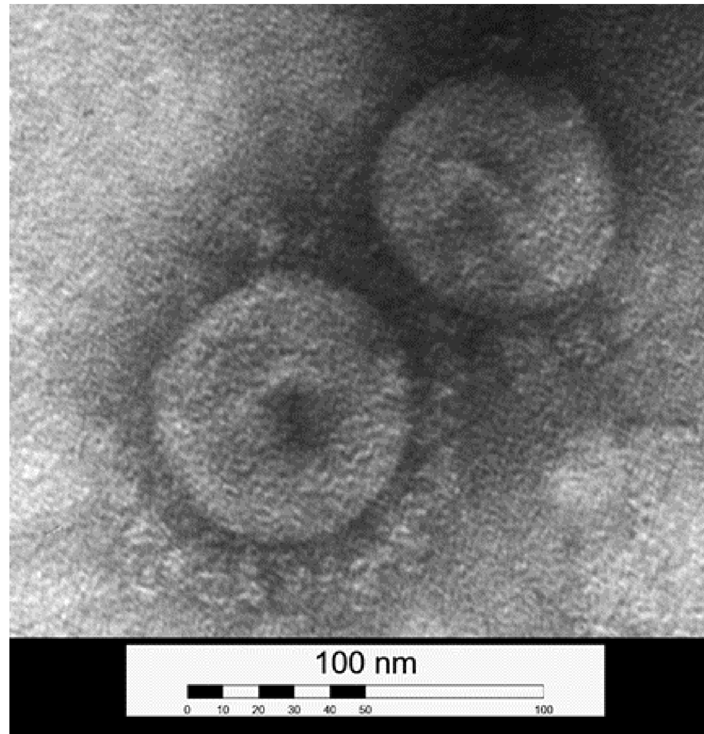

Figure 1. Immune transmission electron microscopic image of canine coronavirus (CCoV)–human pneumonia (HuPn)–2018 from an A72 cell culture. The sample was incubated with anti–CCoV guinea pig serum, leading to the specific viral antibody aggregates. Scale bar represents 100 nm.

findings in other CCoVs [25]. Unlike CCoV I, some FCoV and all betacoronaviruses and gammacoronaviruses, the characteristic multibasic motif (RRXRR)–furin recognition site was absent in the S protein of CCoV-HuPn-2018, suggesting that the virus carries an uncleaved S protein, similarly to most other alphacoronaviruses [15]. Thus, this novel strain shares more similarities with CCoV-II strains.

Surprisingly, there were no unique deletions or insertions in the S protein of CCoV-HuPn-2018. There were also a total of 5 amino acid differences between CCoV-HuPn-2018 and CCoV UCD-1 in the S1 domain; however, these amino acids were identical to those found in the TGEV Purdue S1 and were not unique.

The E protein was 81 amino acids long and did not contain any N-glycosylation sites, whereas 3 N-glycosylated residues have been predicted in each the 261–amino acid M and the 370–amino acid N proteins (Supplementary Figure 3*B* and 3*C*), similar to findings in several other FCoV/CCoV strains. While no evidence of recombination was observed for E, M or N proteins, the N protein contained a unique 12–amino acid deletion within the SR-rich region (located between amino acids 164 and 177 for other CCoV strains). The presence of this deletion was confirmed in the original NSP samples 1116 and 1153.

The 3 ORFs, 3a, 3b, and 3c, between the S and E genes encoded for proteins with sizes of 71, 71, and 244 amino acids, respectively. ORF3, previously found in CCoV I genomes only [14, 25], was not present in the new strain. The 3' end accessory protein gene 7a encoded for 101 amino acids, while there were at least 2 forms of 7b: full-length (213 amino acids) and the one with a 227-nucletide deletion (leading to a frame shift and premature truncation of the putative protein).

DISCUSSION

A previous study identified 8 patients with pneumonia who had molecular evidence of CCoV in their NPS specimens [17]. Partial sequencing and BLASTn analysis suggested that these were closely related but distinct CCoV variants (Supplementary Table 1). The 8 patients with pneumonia were mainly children living in longhouses or villages in rural or suburban areas, where domestic animal and jungle wildlife exposure with the family is common.

In the current study, we confirmed the presence of CCoV with different, less sensitive 1-step RT-PCR assays in 2 specimens, grew a virus in A72 cells from 1 specimen, and conducted a complete genome sequence analysis of the CCoV. Our results demonstrated that CCoV-HuPn-2018 is a novel canine-feline–like recombinant strain with a unique N. To our knowledge, this is the first report suggesting that a CCoV without major genomic rearrangements or adaptive modifications in the S protein might replicate in association with pneumonia in a human host.

Table 3. Complete Genome, Individual Gene Length, and Other Characteristics of Canine Coronavirus–Human Pneumonia–2018

Genomic Region or ORF No.	Coding Sequence	Length, Nucleotides	Putative TRS Start: Nucleotide Position	Putative TRS Start: Sequence	Protein Name	Protein Size, Amino Acids	Note
5′ UTR	No	313	No	No	No	No	Similar to other CCoVs
3′ UTR	No	275	No	No	No	No	
ORF1b	314–20 374	20 061	90	TCGAA*CTAAAC*GAAAT	Pp1ab	6686	Putative ribosomal slippage is at position 12 339
ORF2	20 371–24 717	4347	20 335	GTTA*CTAAAC*TTTG	S	1448	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	AGAA*CTAAAC*TTATG	3a	71	Only 1 TRS before 3a was found; 3a, 3b, and 3c are likely to be expressed from polycistronic mRNAs
ORF3b	24 980–25 195	216	No	No	3b	71	
ORF3c	25 192–25 926	735	No	No	3c	244	
ORF4	25 913–26 158	246	25 866	GGTT*CTAAAC*GAAAT	E	81	No unique features
ORF5	26 169–26 954	786	26 156	TGAA*CTAAAC*AAAAT	M	261	
ORF6	26 967–28 079	1113	26 951	ATAA*CTAAAC*TTCTA	N	370	Contains 36-nucleotide deletion in middle region
ORF7a	28 084–28 389	306	28 072	CGAA*CTAAAC*GAATG	7a	101	Only 1 TRS before 7a is located; 7b is expressed from polycistronic mRNAs
ORF7b	28 394–28 808/29 035	415/642	No	No	7b	34/213	Truncated, likely nonfunctional; contains an out-of-frame 227-nucleotide deletion close to its 5′ end, followed by premature stop codons and full-length forms

Abbreviations: CCoV, canine coronavirus; FCoV, feline coronavirus; mRNAs, messenger RNAs; ORF, open reading frame; TRS, transcription regulatory sequence; UTR, untranslated region.

The conducted analyses demonstrated that the newly identified CCoV-HuPn-2018 was most closely related to CCoV TN-449, while its S1 and S2 domains shared the highest nucleotide identity with CCoV UCD-1 and FCoV WSU 79-1683, respectively. These findings are suggestive of the recombinant nature of this strain, similar to many previously characterized CCoVs [24]. Phylogenetic and recombinational analyses confirmed that CCoV-HuPn-2018 was only distantly related to other *Alphacoronavirus* species, including HCoVs (229E and NL63) and bat CoVs, and likely originated via multiple recombination events between different *Alphacoronavirus 1* strains, but not other alphacoronaviruses. The ability of the novel strain to replicate in A72 canine cells, the absence of ORF3, the higher overall similarity with CCoV-II strains (TN-449 and HLJ-073), and the lack of the furin cleavage site between S1 and S2 domains suggest that the strain belongs to CCoV genotype II [25].

The unique feature not found in any other known CCoVs and *Alphacoronavirus 1* species—namely, the 12–amino acid deletion in the middle portion of the N protein—was confirmed in both original NSP samples, 1153 and 1116. While insertions or deletions in the N protein are not found among the known *Alphacoronavirus 1* strains, the deletion of the SR-rich domain within the middle region of SARS-CoV N protein reportedly resulted in dramatic changes in its cellular localization soon after its zoonotic transmission [26]. Thus, similar to SARS-CoV, CCoV-HuPn-2018 possesses some unique genetic features suggestive of recent zoonotic transmission. Notably, such N protein rearrangements are characteristic of SARS-CoV/SARS-CoV-2 with higher case fatality rates [27].

While SARS-CoV and FCoV NSP7b was not essential for viral replication in vitro and in vivo experiments, its deletion or truncation may be associated with attenuated phenotype [28]. Disruption in the expression of the NSPs after zoonotic transmission of SARS-CoV was reported previously, suggesting that it may represent an adaptive mechanism [29]. Finally, deletions unique to FIPVs were found in ORFs 3c and/or 7b and were hypothesized to be responsible for the shift from enteric (FCoV) to FIPV phenotype and increased pathogenicity [30]. The ability of CCoV to evolve quickly through frequent recombination events and induce disease of variable severity is even more concerning, given that these data indicating that circulating CCoV may already be transmittable to humans.

The current study had a number of limitations. First, we have not met recognized standards of causality, such as Koch postulates or Bradford Hill criteria. Second, we recognize that the detected CCoVs could only be “carried” in some of the 8 patients’ airways, not causing disease. However, identification of (1) FCoV-like CoVs in influenza-negative patients with acute respiratory symptoms in Arkansas and (2) porcine deltacoronavirus in children in Haiti further emphasizes that *Alphacoronavirus 1* species may be infectious or pathogenic to humans [18, 31].

In conclusion, we recovered and characterized a novel recombinant CoV, CCoV-HuPn-2018, from a hospitalized patient

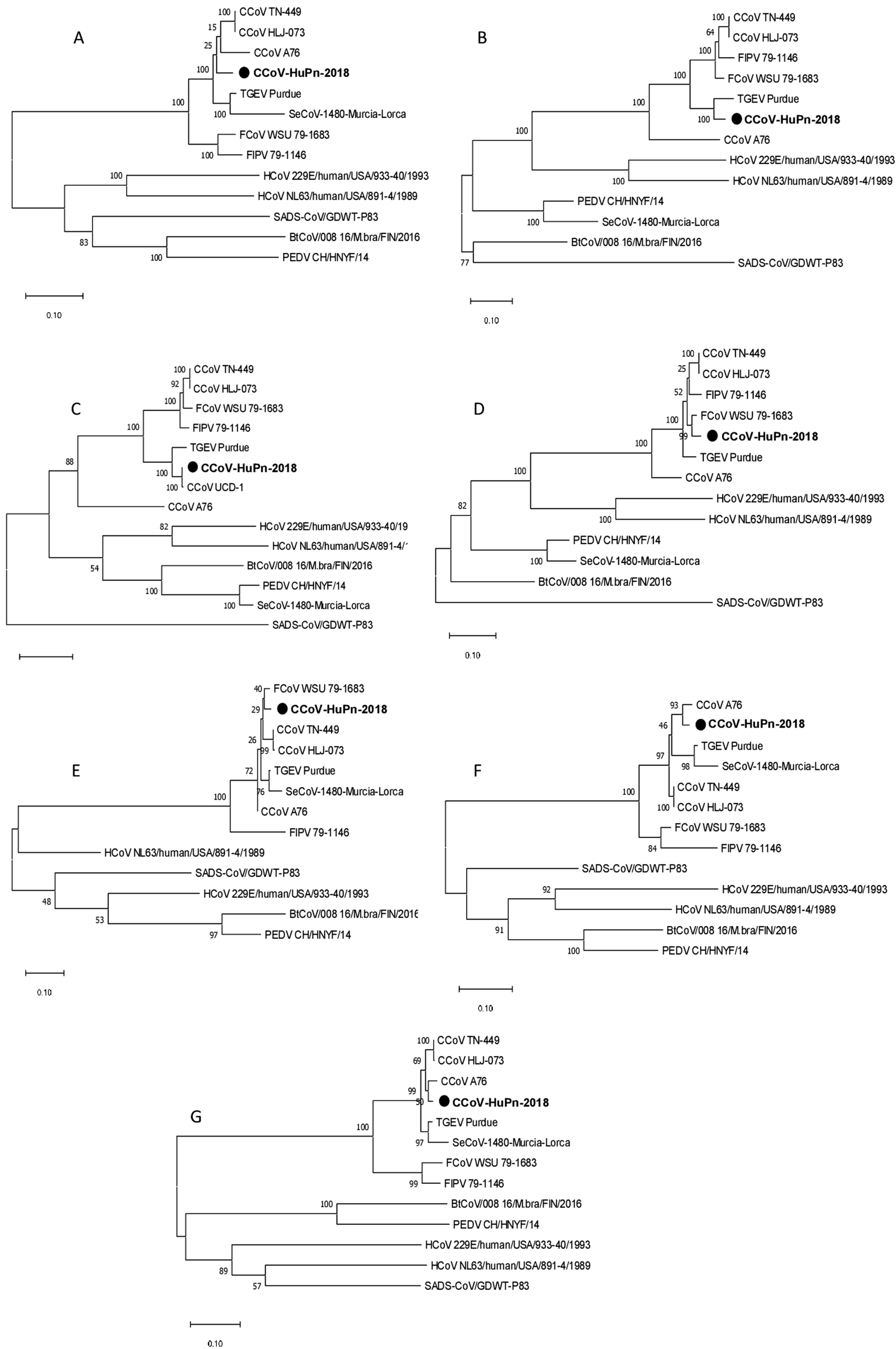

Figure 2. Phylogenetic tree based on complete genome (*A*), S gene (*B*), S1 (*C*), S2 domain (*D*), E gene (*E*), M gene (*F*) and N gene (*G*) sequences of the canine coronavirus (CCoV)–human pneumonia (HuPn)–2018 viral isolate and other *Alphacoronavirus* species. Bootstrap values are represented at key nodes. Scale bar indicates nucleotide substitutions per site. The evolutionary history was inferred using the maximum likelihood method and the general time-reversible model. This analysis involved 13 nucleotide sequences. Evolutionary analyses were conducted using MEGA X software. Black circles represent the newly identified viral isolate, CCoV-HuPn-2018. Abbreviations: BtCoV, bat coronavirus (CoV); FCoV, feline CoV; FIPV, feline infectious peritonitis virus; HCoV, human CoV; PEDV, porcine epidemic diarrhea virus; SADS-CoV, swine acute diarrhea syndrome CoV; SeCoV, Swine enteric CoV; TGEV, transmissible gastroenteritis virus.

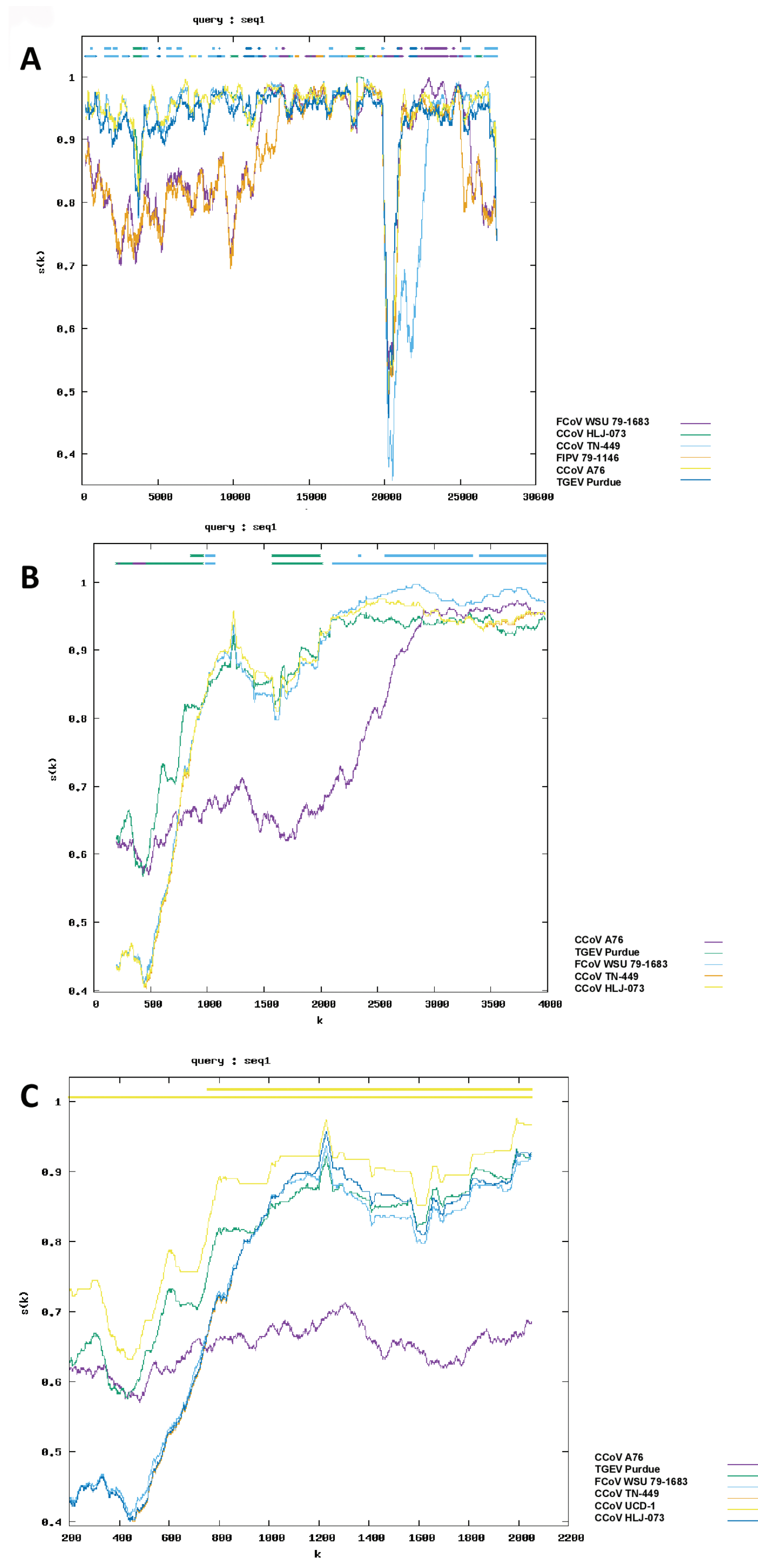

Figure 3. Recombinational analysis of the canine coronavirus (CCoV)–human pneumonia (HuPn)–2018 complete genome (*A*), S1 (*B*) and S2 (*C*) domains. At each position of the window, the query sequence CCoV-HuPn-2018 was compared with background sequences for 6 strains shown in the legend on the right. The x-axes represent the length of the sequence, and the y-axes, the similarity value (Similarity = Match Fraction = 1 - distance). The two bars on the top of the graph represent the "best match" (lower bar), and the significance of this match (upper bar). The "best match" sequence is the background sequence with the highest similarity to the query. The upper bar is also colored at a position when the best match is significantly better than the second match. Arrows represent potential recombination break points. Abbreviations: FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; TGEV, transmissible gastroenteritis virus.

with pneumonia. While possessing some unique characteristics likely suggestive of a recent zoonotic transmission, this novel strain with recombinant CCoV UCD-1/FCoV WSU 79-1683 S protein shares multiple genomic features of widespread CCoV-II. Further studies are needed to investigate CCoV prevalence, seroprevalence, and pathogenic potential in humans. Additional studies should be conducted to evaluate the biological relevance of the observed deletion in the N protein.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. The authors thank King-Ching Hii, Jane K. Fieldhouse, Jakie Ting, Antoinette Berita, Tham Thi Nguyen, See-Chang Wong, Toh-Mee Wong, Wei-Honn Lim, Siaw-Jing Ha, Chuet-Zou Lau, Sing-Ling Kong, Emily S. Bailey, Mohd Raili Suhaili, Kristen K. Coleman, Son Thế Than, Tyler E. Warkentien, Patrick J. Blair, Nga-Hung Ngu, Khai-Fatt Chao, Cheng-Ing Kong, Zhen-Hao Chin, Edmund Kwang-Yuen Wong, Tiana Ti, Hilary Hon-Yun Kueh, Cornelius Jambol, Goh Hieng-Hua Goh, Velarie Bill, Tiing-Tiing Chua, Raquel A. Binder, Raquel A. Natalie A. Alarja, Emily R. Robie, Anfal Abdelgadir, and John A. Lednicky for their clinical, laboratory, scientific, and administrative contributions to this work.

This research was conducted in partnership with Duke University, the Duke Global Health Institute, Sibu Hospital Clinical Research Center, SEGi University Sibu Clinical Campus, and The Ohio State University. The authors also thank Tea Meulia (Molecular and Cellular Imaging Center of the Ohio Agricultural Research and Development Center) for assistance with transmission electron microscopy.

The study—source of nasopharyngeal swab samples—has received a scientific review, and all procedures followed were in accordance with the ethical standards of the Malaysian Ministry of Health's Medical Research and Ethics Committee (protocol NMRR-17-316-34395), the Duke University Health System Institutional Review Board, the Duke-NUS Medical School Ethical Review Board, and the Naval Medical Research Center–Asia Human Research Protection Program (HRPO no. W911QY-16-D-0058).

Author Contributions. A. N. V. designed, oversaw, and provided financial support for the experiments on canine coronavirus (CCoV)–human pneumonia (HuPn)–2018 characterization and sequences, sequenced parts of the genome, analyzed the data, and wrote the manuscript. A. D. conducted most of the experiments on CCoV-HuPn-2018 cell culture isolation and Sanger sequencing. D. D. conducted some of the experiments on Sanger sequencing. L. X. screened 301 samples and identified the 8 samples positive for coronavirus/CCoV. T. H. T. and J. S. Y. L. coordinated sample collection, obtained ethical clearance, and collected and processed the patient demographic data. L. J. S. critically revised the manuscript draft. G. C. G. led the original studies, oversaw the new study, provided financial support, and revised the manuscript.

Financial support. This work was supported by the US Naval Medical Research Center–Asia and Vysnova Partners (grants SC-2016-SABER-003-002 and SC-2017- SABER-010-001 to G. C. G.), Duke University's Global Health Institute (discretionary funds to G. C. G.), and The Ohio State University (start-up funds to A. N. V.).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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