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

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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.
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 3x coverage, using the Sanger dye-deoxy 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 ampiclon-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 polycistrionic 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
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 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 3A), similar to

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

<table>
<thead>
<tr>
<th>Alphacoronavirus 1</th>
<th>Strain</th>
<th>Accession No.</th>
<th>Complete Genome</th>
<th>S</th>
<th>S1</th>
<th>S2</th>
<th>E</th>
<th>M</th>
<th>N</th>
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<td>CCoV-IIa</td>
<td>TN-449</td>
<td>JQ404410.1</td>
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<td>93.42</td>
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<td>95.20</td>
<td>93.57</td>
<td>95.08</td>
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<td>CCoV-IIa</td>
<td>HLJ-073</td>
<td>KY063618.2</td>
<td>91.74</td>
<td>93.33</td>
<td>73.32</td>
<td>95.20</td>
<td>93.17</td>
<td>95.08</td>
<td>93.33</td>
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<tr>
<td>CCoV-IIc</td>
<td>A76</td>
<td>JN856008.2</td>
<td>90.63</td>
<td>93.77</td>
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<td>85.42</td>
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<td>97.08</td>
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<td>AF116248.1</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
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<td>92.12</td>
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<tr>
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<td>JN834064.1</td>
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<td>DQ010921.1</td>
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<td>95.04</td>
<td>79.92</td>
<td>81.77</td>
<td>75.5</td>
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</table>

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.

*Highest nucleotide identity between CCoV-HuPn-2018 and given strain.
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 3B and 3C), 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-nucleotide 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.
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 will 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

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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 reviewed the manuscript draft. G. C. G. led the original studies, oversaw the new study, provided financial support, and revised the manuscript.

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