

Detection and Characterization of New Coronavirus in Bottlenose Dolphin, United States, 2019

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We characterized novel coronaviruses detected in US bottlenose dolphins (BdCoVs) with diarrhea. These viruses are closely related to the other 2 known cetacean coronaviruses, Hong Kong BdCoV and beluga whale CoV. A deletion in the spike gene and insertions in the membrane gene and untranslated regions were found in US BdCoVs (unrelated to severe acute respiratory syndrome coronavirus 2).

The coronavirus family consists of single-stranded, positive-sense RNA viruses that cause respiratory, gastrointestinal, hepatic, and neurologic diseases of different host species. On the basis of genetic characterization, coronaviruses have been classified into 4 genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. *Cetacean coronavirus* is a recently proposed new species in the genus *Gammacoronavirus*, in addition to a common species (*Avian coronavirus*) (1). *Cetacean coronavirus* species contains bottlenose dolphin coronavirus (BdCoV) HKU22, identified in 2014, and beluga whale coronavirus (BWCoV) SW1, identified in 2008 (1,2). We report detection and genetic characterization of BdCoV in bottlenose dolphins in the United States; all dolphins had clinical signs consistent with gastrointestinal discomfort.

Four Atlantic bottlenose dolphins cared for by the US Navy Marine Mammal Program (San Diego, CA) showed development of an acute onset of clinical illness with clinical signs consisting of inappetence (n = 4), diarrhea (n = 3), and lethargy (n = 2) during April and May 2019. We collected fecal samples as part of the minimum workup for acute illness. Among all viruses we tested by using

conventional PCRs, only coronavirus showed a positive result for all 4 dolphins.

We further evaluated samples by using next-generation sequencing as described (3). De novo assembly analysis of raw FASTQ data showed that 4 near complete genomes of BdCoV were assembled. Gaps were closed by Sanger sequencing at ACGT, Inc. (<https://www.acgtinc.com>). The genomes of all 4 US BdCoVs (37112-1, -2, -3, and -4) comprised 31,728 nt (GenBank accession nos. MN690608-11), which were shorter than those of 3 Hong Kong BdCoVs (HK-BdCoVs) (31,750-31,758 nt).

Further analysis of all individual genes showed that the 4 US BdCoV strains showed similar identities to both HK-BdCoVs and BWCoV in open reading frame (ORF) 1a, ORF1b, nonstructural (NS) 7, NS8, NS9, and NS10. However, US BdCoV strains showed higher identities to HK-BdCoVs than to BWCoV only in spike (S), envelope (E), membrane (M), and NS5a instead of all remaining genes (Appendix Table, Figure 1, <https://wwwnc.cdc.gov/EID/article/26/7/20-0093-App1.pdf>).

The 4 US BdCoV strains showed relatively higher identities to BWCoV than to HK-BdCoVs in NS5b (95.9% vs. 93.8%-94.0%), NS5c (98.4% vs. 97.7%-97.9%), NS6 (94.9% vs. 88.6%-88.9%), and nucleocapsid protein (97.9% vs. 96.1%-96.5%) genes. Analysis of amino acid identities of different ORFs also showed similar patterns (Appendix Table).

Phylogenetic analysis of complete genomes showed that the 4 US BdCoVs were clustered with 3 HK-BdCoVs strains and distantly related to BdCoV SW1 but were distinct from avian coronaviruses (Figure). Phylogenetic trees for individual genes showed that US BdCoV strains have a greater correlation with HK-BdCoVs than BWCoV in the S, E, M, NS5a, and NS7 genes and to BWCoV than HK-BdCoVs in remaining genes (Figure; Appendix Figures 2, 3).

Compared with the Hong Kong CF090331 strain, all US BdCoV strains have a 42-nt deletion in the S1 region at positions 21366-21407 encoding an S protein that is 14-aa shorter (Appendix Figure 4). Compared with 3 HK-BdCoVs strains, a 3-nt insertion (ACA) at positions 25417-25419 was found in the M gene of the 4 US BdCoVs, leading to a frameshift mutation in the M protein that was 1 amino acid longer (Appendix Figure 5).

In addition, a 4-nt insertion (TATA) was found in the 5' untranslated region (UTR) of US BdCoV strains, and a 1-nt insertion (T) was found in the 3' UTR of US BdCoV strains (Appendix Figure 6). Similar to findings of a previous report (1), because of 1 nt mutation (G→T at position 28268) of the US BdCoV strains, a premature stop codon in the NS7 gene resulted in

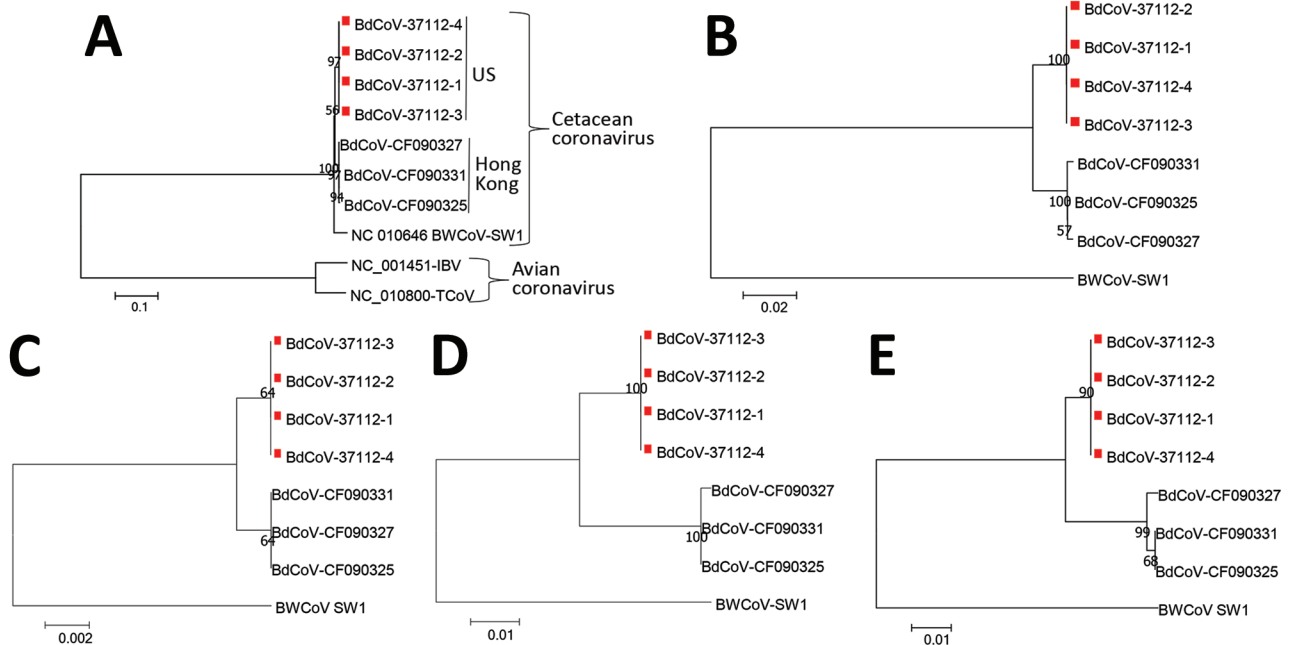


Figure. Phylogenetic analysis of A) complete genome, B) spike, C) envelope, D) matrix, and E) nonstructural protein 5a genes of gammacoronaviruses, including 4 US BdCoVs, 37112-1 to -4 (GenBank accession nos. MN690608–MN690611, indicated with red squares); 3 Hong Kong BdCoVs (accession nos.: CF090327, KF793825; CF090331, KF793826; CF090325, KF793824); and 1 BWCov (SW1, accession no. NC_010646). Numbers along branches are bootstrap values. Scale bars indicate nucleotide substitutions per site. BdCoV, bottlenose dolphin coronavirus; BWCov, beluga whale coronavirus.

an NS7a (42 aa) and an NS7b (117 aa). The position of the premature stop codon in the US BdCoVs was different from that for 2 HK-BdCoVs (CF090325 and CF090331), which encode different sizes of NS7a (63 aa) and NS7b (34 aa) proteins.

S deletion variants are commonly observed in coronaviruses. Porcine respiratory coronavirus is an S gene deletion mutant of transmissible gastroenteritis virus and causes a respiratory disease instead of gastroenteritis in pigs because of a large deletion (>600 nt) in the N terminal of the S gene (4). In addition, a large deletion (591 nt) in S1 resulted in a change in virulence of porcine epidemic diarrhea virus (5). In our study, we observed that 4 US BdCoVs had a 42-nt deletion in the S1 gene. It is unclear whether this deletion region is related to the viral tropism and virulence, and warrants further studies.

During a surveillance study in Hong Kong, China, BdCoV was identified only in fecal samples from dolphins that had no notable clinical signs (1). In our study, genetically related BdCoVs were detected in dolphins that had diarrhea, lethargy, and inappetence in the United States. It is possible that unique genetic features of US BdCoVs, including a sequence deletion in the S gene and an insertion in the M gene and 5' and 3' UTRs, and mutations in different genes might have

contributed to the observed clinical diarrhea signs in US dolphins. Additional surveillance is needed to monitor the evolution of this virus worldwide.

About the Author

Dr. Wang is a clinical assistant professor at the College of Veterinary Medicine, University of Illinois, Urbana. His research interests are diagnosis of viral infectious diseases and novel pathogen discovery.

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Human Case of Severe Fever with Thrombocytopenia Syndrome Virus Infection, Taiwan, 2019

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We report on a 70-year-old man with fever, leukopenia, thrombocytopenia, vomiting, malaise, dyspnea, and consciousness disturbance who was infected with severe fever with thrombocytopenia syndrome virus in northern Taiwan, 2019. This autochthonous case was confirmed by reverse transcription PCR, virus isolation, and genomic sequencing.

Severe fever with thrombocytopenia syndrome (SFTS) is a tickborne infection caused by the SFTS virus (SFTSV, also known as *Huaiyangshan banyangvirus*), which was identified in China in 2009 (1) and afterward in South Korea (2), Japan (3), and Vietnam

(4). Since then, the number of SFTS cases in East Asia has risen rapidly. Therefore, laboratory-based surveillance of SFTS has been conducted in the routine molecular diagnosis of arboviral infections in the Taiwan Centers for Disease Control (Taiwan CDC) since 2013. We identified a patient in Taiwan with laboratory-confirmed SFTS who was originally suspected of having dengue or rickettsial infections.

In November 2019, a 70-year-old man who lived in northern Taiwan and had no travel history was admitted to the hospital with a 9-day history of fever (38.8°C–39.2°C), chills, nausea, vomiting, and malaise. The patient had underlying hypertension and type 2 diabetes mellitus that was controlled without medication. At hospital admission, we noted a generalized rash over the trunk and both feet. Laboratory examinations showed that the patient had leukopenia; thrombocytopenia; abnormal prothrombin time; elevated levels of aspartate transaminase, alanine transaminase, creatinine kinase, and C-reactive protein; and diagnostic disseminated intravascular coagulation (Table). Chest radiography and chest computed tomography showed patchy consolidations and ground-glass opacities of both lungs. A few hours after admission, the patient experienced a general tonic-clonic seizure, with worsening consciousness and dyspnea. He was transferred to the intensive care unit, where intubation and ventilator support began. He also received massive blood transfusions for severe thrombocytopenia, active mucosal (oral, nasal, and gastrointestinal tract) bleeding, and disseminated intravascular coagulation. Blood and sputum cultures revealed that the patient was infected with *Pseudomonas aeruginosa*; he received piperacillin/tazobactam, doxycycline, and clarithromycin as empirical therapy. Results of laboratory tests for hepatitis A and B viruses, cytomegalovirus, herpes simplex virus, adenovirus, and influenza were all negative. After the patient received a diagnosis of SFTSV infection, he received treatment with intravenous immunoglobulin for 5 days. However, his condition continued to deteriorate progressively. The patient died on day 40 after illness onset as a result of multiorgan failure. Delayed diagnosis and the presence of underlying conditions in this patient, including hypertension and diabetes mellitus, may be associated with his severe disease and death (5).

The patient often spent time on a vegetable farm in a mountainous area without wearing shoes, raising suspicions for arboviral and rickettsial infections. The hospital sent blood samples, collected from the patient before the blood transfusions on day 12 after illness onset, to the Taiwan CDC for

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Appendix

Cetacean Coronavirus

The major differences between HKU22 and SW1 were found in their spike (S) and membrane genes; only 74.3%–74.7% and 85.5% amino acid identities, respectively (*1*). Beluga whale coronavirus (BwCoV) SW1 was detected in liver tissue from a deceased whale, and bottlenose dolphin CoV (BdCoV) HKU22 was detected during routine surveillance study in which no dolphins showed any notable signs. Because of having unique open reading frames (ORFs) (nonstructural [NS]5a, NS5b, NS5c, NS6, NS7, NS8, NS9, and NS10) between membrane and nucleocapsid genes, both bottlenose dolphin CoV HKU22 and beluga whale CoV SW1 have the largest genome sizes among all CoVs.

Animal Care

Fecal samples were collected at the US Navy Marine Mammal Program (MMP) during routine care of dolphins and under US Code, Title 10, USC 7524. The MMP houses and cares for a population of bottlenose dolphins in San Diego Bay (CA, USA). MMP is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and adheres to the national standards of the US Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Animal Welfare Act. The Navy Marine Mammal Program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and adheres to the national Humane Care and Use of Laboratory Animals and the Animal Welfare Act. As required by the Department of Defense, the Program's animal care and use program is routinely reviewed by an Institutional Animal Care and Use Committee and the Department of Defense Bureau of Medicine.

Viral Testing

Fecal samples of all dolphins were tested by using conventional PCR methods. Samples showed negative results for paramyxovirus, rotavirus, canine distemper virus, canine parvovirus, and adenovirus (Athens Veterinary Diagnostic Laboratory, University of Georgia, Athens, GA, USA).

Bacterial Testing

In addition to those viral testing listed in the main text, 4 dolphins were tested for different bacteria (*Campylobacter*, *Salmonella*, and *Lawsonia* species) and toxins of *Escherichia coli* (attaching and effacing toxin, cytolethal distending toxin, and cytotoxic necrotizing factor 1) and *Clostridium perfringens* (alpha toxin, enterotoxin, and beta 2 toxin). Results showed that 2 dolphins were positive for *E. coli* cytotoxic necrotizing factor 1, and 1 dolphin was positive for alpha toxin and entero toxin of *C. perfringens*.

Next-Generation Sequencing

Nucleic acids extracted from the fecal samples were subjected to a sequence-independent, single-primer amplification and library preparation by using a Nextera XT Kit (<https://www.illumina.com>) and sequencing by using MiSeq Reagent Kit version 2 (Illumina) (500 cycles) as described (2). Raw sequence data were submitted to the Sequence Read Archive under BioProject no. PRJNA612626.

Results

Raw FASTQ (Illumina) data for 4 dolphin samples were analyzed by using Kraken 2 and a standard Kraken database (3). Analyses showed that 99%–100% of viral reads of all 4 samples (37112–1, –2, –3, and –4) are CoV reads, and no other viruses previously tested by conventional PCR were observed. The genome organization and the transcription regulatory sequence motif 5'-AAACA of US BdCoVs are the same as those for Hong Kong BdCoVs and BdCoV SW1 (Appendix Figure 1). Alignment analysis of the complete nucleotide genome showed that 4 BdCoV strains (37112–1, –2, –3, and –4) were closely related to each other, had high identities (99.9%), and showed 97% and 94% identities with 3 Hong Kong BdCoVs and BdCoV SW1 strain, respectively, but showed low identities (46%) with avian CoVs. On the basis of the 90%

amino acid identity threshold for a new species, 4 US BdCoVs belong to the species *Cetacean CoV*.

Compared with the Hong Kong CF090331 strain, the area of the S deletion in US BdCoV seems to be a variable region. However, the Hong Kong strain CF090325 has a 9-nt deletion, and Hong Kong strain CF090325 has a 12-nt deletion.

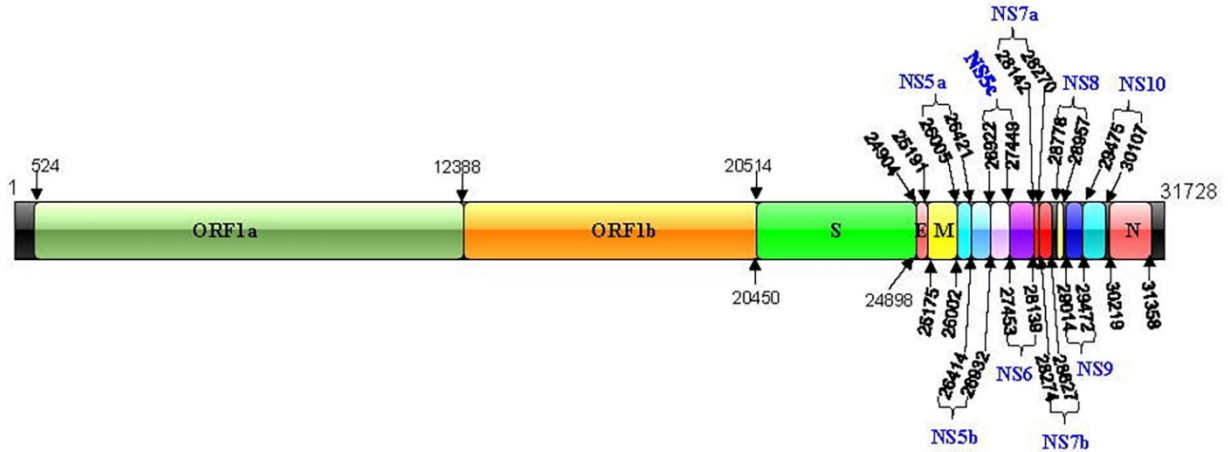
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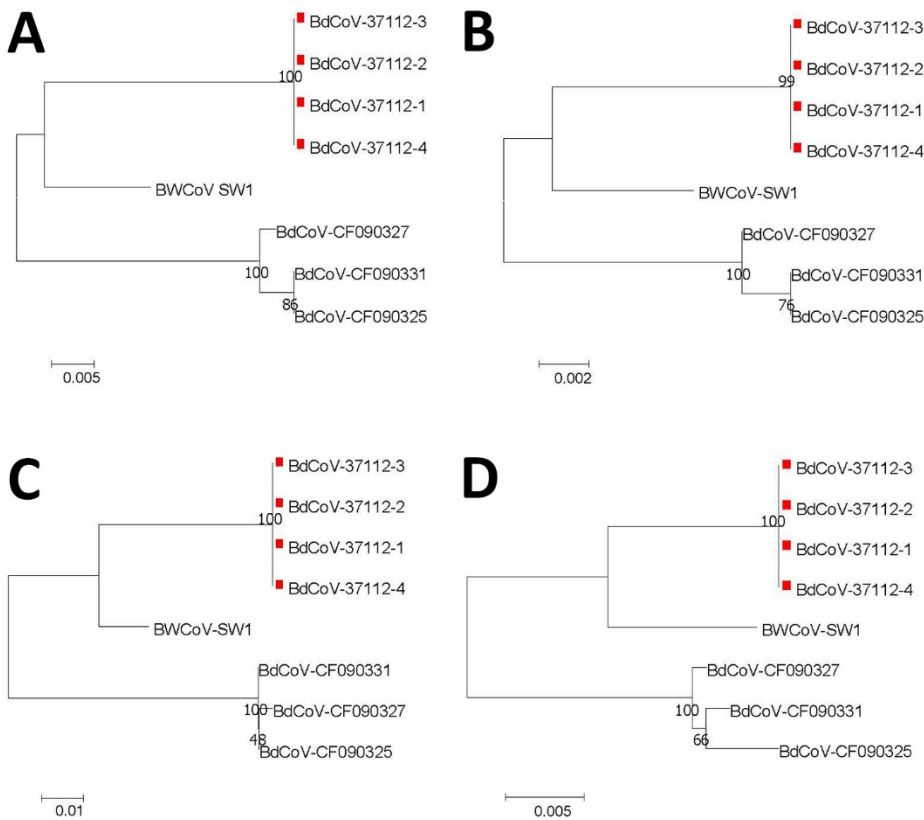
Appendix Table. Nucleotide and amino acid identities of US BdCoV strains (37112–1 to –4) with other marine mammal CoVs, including 3 Hong Kong BdCoVs and 1 Bw CoV SW1*

Gene	Nucleotide identity, %				Amino acid identity, %			
	Hong Kong BdCoVs			Bw CoV	Hong Kong BdCoVs			Bw CoV
	CF090325	CF090327	CF090331	SW1	CF090325	CF090327	CF090331	SW1
ORF								
1a	97.8	97.9	97.9	97.8	97.9–98.0	98.2	98.1	98.2–98.3
1b	98.2	98.4	98.2–98.3	98.3–98.4	99.2–99.3	99.4–99.5	99.3–99.4	99.1–99.2
S	96.4	96.4	96.1	75.9	96.7	96.4	96.1	74.8
E	99.3	99.3	99.3	95.1	98.9	98.9	98.9	96.8
M	93.6	93.3	93.6	83.5	96.0	95.2	96.0	85.1
NS5a	97.1	97.1	97.1	88.7	98.5	98.5	98.5	92.0
NS5b	93.8	94.0	93.8	95.9	93.0	92.4	93.0	95.3
NS5c	97.7	97.9	97.7	98.4	97.7	98.2	97.7	97.7
NS6	88.9	88.6	88.9	94.9	91.6	91.6	91.6	95.1
NS7	94.6	94.8	94.6	94.2	–	–	–	–
NS8	97.7	97.2	97.7	97.7	96.6	94.9	96.6	96.6
NS9	98.0–98.2	97.8–98.0	98.0–98.2	98.0–98.2	96.7–97.3	96.7–97.3	96.7–97.3	96.7–97.3
NS10	99.0	98.2	99.0	98.4	99.0	97.1	99.0	99.0
N	96.1	96.5	96.4	97.9	96.0	97.0	97.0	99.7

*BdCoV, bottlenose dolphin coronavirus; BwCoV, Beluga whale coronavirus, E, envelope; M, membrane; N, nucleocapsid; NS, nonstructural; ORF, open reading frame; –, amino acid identity of NS7 gene was not determined because of a premature stop codon at different locations between US BdCoVs and Hong Kong BdCoVs.

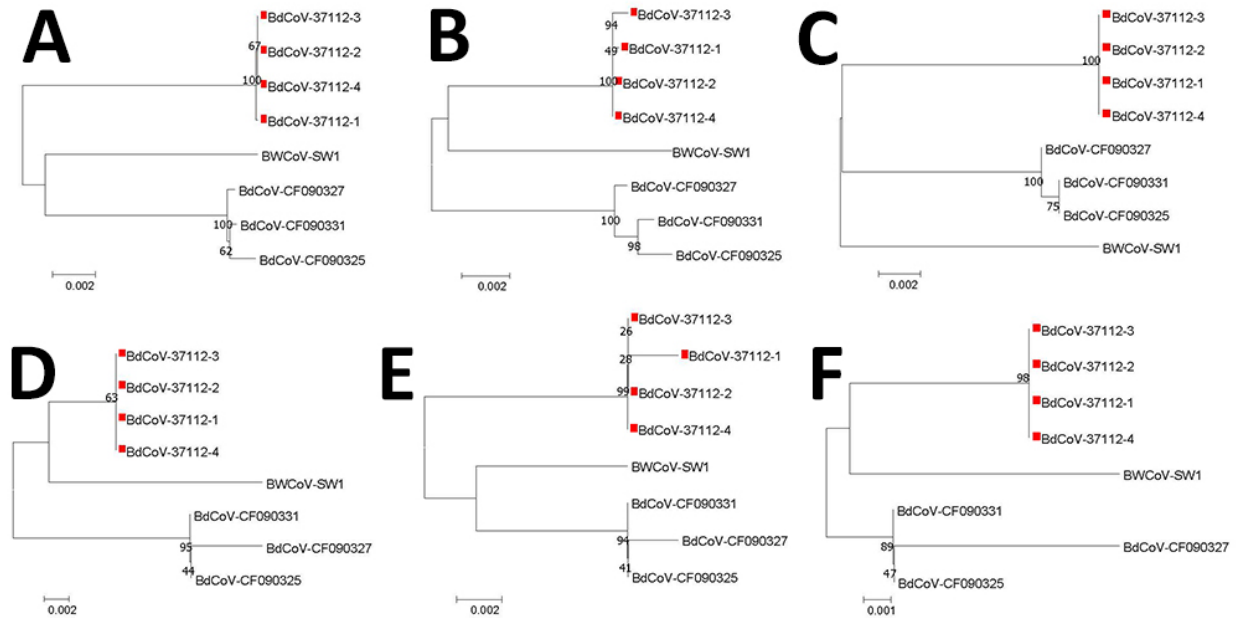


Appendix Figure 1. Schematic diagram of bottlenose dolphin coronavirus genome of US strains (37112–1 to –4). E, envelope; M, membrane, N, nucleocapsid; NS, nonstructural; ORF, open reading frame; S, spike.

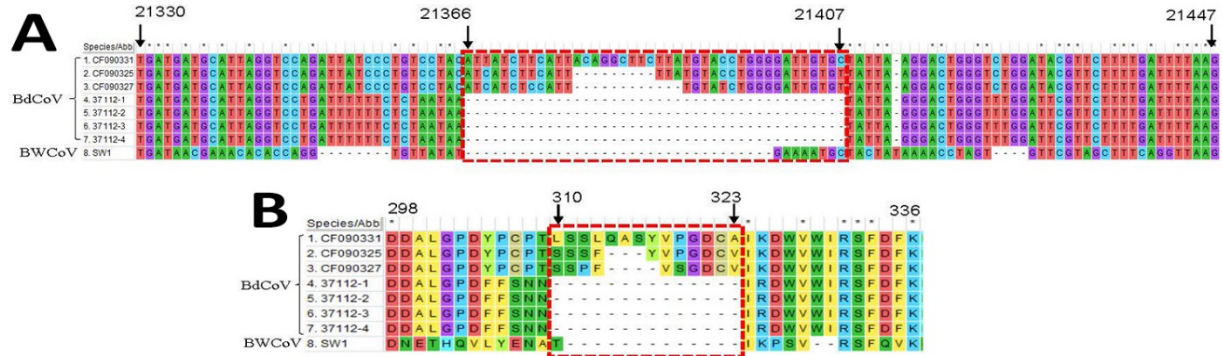


Appendix Figure 2. Phylogenetic tree analysis of NS5b, NS5c, NS6, and N genes of gammacoronaviruses, including 4 US BdCoVs (GenBank accession nos. MN690608–11, indicated by a red square), 3 Hong Kong BdCoVs (CF090327: KF793825; CF090331: KF793826; CF090325:

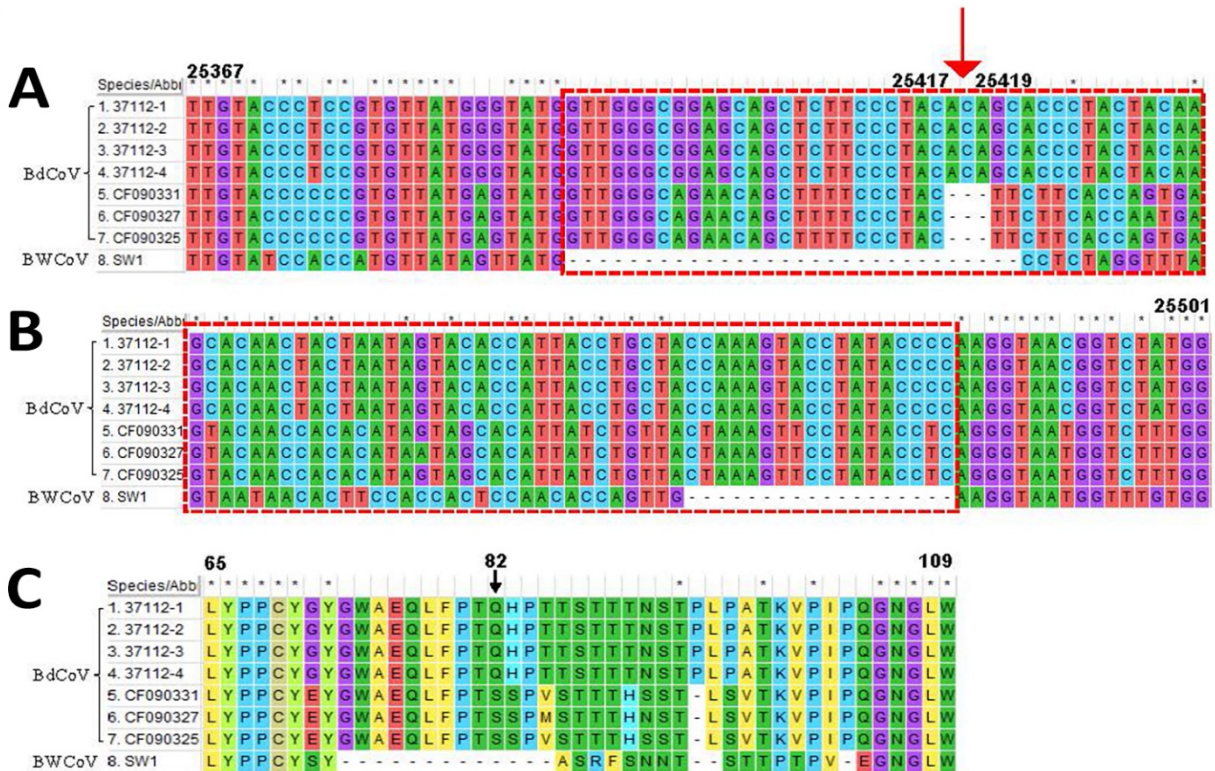
KF793824), and 1 BwCoV (SW1: NC_010646). A) NS5b, B) NS5c, C) NS6, D) N. Scale bars indicate nucleotide substitutions per site. BdCoV, bottlenose dolphin coronavirus; BwCoV, Beluga whale coronavirus, N, nucleocapsid; NS, nonstructural.



Appendix Figure 3. Phylogenetic tree analysis of A) ORF1a, B) ORF1b, C) NS7, D) NS8, E) NS9, and F) NS10 genes of gammacoronaviruses, including 4 US. BdCoVs (GenBank accession nos. MN690608–11, indicated with a red square), 3 Hong Kong BdCoVs (CF090327: KF793825; CF090331: KF793826; CF090325: KF793824), and 1 BwCoV (SW1: NC_010646). Scale bars indicate nucleotide substitutions per site. BdCoV, bottlenose dolphin coronavirus; BwCoV, Beluga whale coronavirus, ORF, open reading frame; NS, nonstructural.



Appendix Figure 4. Nucleotide and amino acid sequence alignment of partial S gene of coronaviruses at positions 21330–21447 by using MUSCLE in MEGA 7.0.26 (<https://www.megasoftware.net>). Deletion region is indicated by a red color frame between positions 21366 and 21407 of the nucleotide sequence and between positions 310–323 of amino acid sequence. Labeled positions were based on CF090331 (GenBank accession no. KF793826). *, same nucleotide or amino acid; –, deletion. BdCoV, bottlenose dolphin coronavirus; BwCoV, Beluga whale coronavirus; S, spike.



Appendix Figure 5. Nucleotide and amino acid sequence alignment of partial M gene of coronaviruses at positions 25367–25501 by using MUSCLE in MEGA 7.0.26 (<https://www.megasoftware.net>). Insertion region was between positions 25417 and 25419 of the nucleotide sequence, which caused frameshift

