BRIEF REPORT

Genomic and serological detection of bat coronavirus from bats in the Philippines

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Abstract Bat coronavirus (BtCoV) is assumed to be a progenitor of severe acute respiratory syndrome (SARS)-related coronaviruses. To explore the distribution of BtCoVs in the Philippines, we collected 179 bats and detected viral RNA from intestinal or fecal samples by RT-PCR. The overall prevalence of BtCoVs among bats was 29.6 %. Phylogenetic analysis of the partial RNA-dependent RNA polymerase gene suggested that one of the detected BtCoVs was a novel alphacoronavirus, while the others belonged to the genus *Betacoronavirus*. Western blotting revealed that 66.5 % of bat sera had antibodies to BtCoV. These surveys suggested the endemic presence of BtCoVs in the Philippines.

Abbreviations

BtCoV	Bat coronavirus
CoV	Coronavirus
SARS	Severe acute respiratory syndrome
RT-PCR	Reverse transcriptase polymerase chain reaction
RdRp	RNA–dependent RNA polymerase
nt	Nucleotide
N	Nucleocapsid
PBS	Phosphate-buffered saline
WB	Western blotting
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
HRP	Horseradish peroxidase

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Recently, bats have been recognized as a major reservoir of emerging viral infections that cause serious diseases in humans and other mammals [1]. Several viruses that cause severe diseases in humans have been detected in bats, such as lyssaviruses related to rabies virus, Hendra virus, Nipah virus, Ebola virus, Marburg virus and severe acute respiratory syndrome (SARS) coronavirus [1]. Hence, there is a growing focus on bats as zoonotic hosts.

SARS was caused by a previously unknown coronavirus [2]. From 2002 to 2003, the SARS epidemic affected mainly China, Hong Kong and Taiwan, afflicting about 8000 people and killing over 600 [3]. Investigations of bats revealed that horseshoe bats (*Rhinolophus*) in southern China were natural reservoirs of a coronavirus related to SARS-CoV [4, 5].

This prompted surveys of bat coronaviruses (BtCoVs) worldwide, and novel viruses were detected in North and South America, Africa, Europe and Asia [6–12], revealing a broad diversity of BtCoVs. Molecular phylogenetic analysis suggested that all mammalian coronaviruses including SARS-CoV were likely to have evolved from BtCoVs [13].

We previously reported that two groups of BtCoVs were detected in the Philippines: one belonging to the genus *Alphacoronavirus* and the other belonging to the genus *Betacoronavirus* [14]. To explore the diversity of BtCoVs in the Philippines, we carried out more surveys in this study. We investigated 179 intestinal or fecal samples to detect coronaviral RNA and performed phylogenetic analysis on isolated coronavirus sequences. We also detected virus-specific antibodies, indicating how widely distributed BtCoVs are in the Philippines.

Table 1 Prevalence of coronavirus in bat fecal samples detected by RT-PCR and WB analysis

Year	Location	Species	RT-PCR positive	WB positive	Samples tested
2009[#]	Site A	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	1	1	2
		Greater musky fruit bat (*Ptenochirus jagori*)	5	10	10
		Philippine sheath-tailed bat (*Emballonura alecto*)	2	5	8
		Large rufous horseshoe bat (*Rhinolophus rufus*)	1	2	2
		Philippine pygmy fruit bat (*Haplonicteris fischeri*)	0	1	6
		Lesser long-tongued fruit bat (*Macroglossus minimus*)	0	1	2
		Total	9	20	30
	Site B	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	1	4	6
		Greater musky fruit bat (*Ptenochirus jagori*)	1	6	8
		Geoffroy rousette bat (*Rousettus amplexicaudatus*)	1	4	5
		Total	3	14	19
2010*	Site C	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	16	32	43
		Greater musky fruit bat (*Ptenochirus jagori*)	1	6	11
		Geoffroy rousette bat (*Rousettus amplexicaudatus*)	0	0	1
		Diadem roundleaf bat (*Hipposideros diadema*)	0	0	3
		Total	17	38	58
	Site D	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	11	17	28
		Greater musky fruit bat (*Ptenochirus jagori*)	4	14	21
		Diadem roundleaf bat (*Hipposideros diadema*)	1	0	1
		Total	16	31	50
2011*	Site E	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	2	4	4
		Greater musky fruit bat (*Ptenochirus jagori*)	5	12	17
		Lesser long-tongued fruit bat (*Macroglossus minimus*)	1	0	1
		Total	8	16	22
Total		Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	31	58	83
		Greater musky fruit bat (*Ptenochirus jagori*)	16	48	67
		Philippine sheath-tailed bat (*Emballonura alecto*)	2	5	8
		Large rufous horseshoe bat (*Rhinolophus rufus*)	1	2	2
		Philippine pygmy fruit bat (*Haplonicteris fischeri*)	0	1	6
		Lesser long-tongued fruit bat (*Macroglossus minimus*)	1	1	3
		Geoffroy rousette bat (*Rousettus amplexicaudatus*)	1	4	6
		Diadem roundleaf bat (*Hipposideros diadema*)	1	0	4
		Total	53	119	179

intesteinal and * fecal samples were examined by RT-PCR

A total of 179 bats (8 species) were captured at five different sites in the Philippines during the months of March 2009, August 2010 and May 2011. Each sampling was performed after receiving permission from the Government of the Philippines. For anesthesia, 15 mg/kg tiletamine and zolazepam (Virbac, Carros, France) were injected intraperitoneally into captured bats, and the anesthetized bats were sacrificed by cardiac exsanguination. Liver, lienal, renal, lung, fecal and intestinal specimens were collected from each captured bat and were shared with the coworkers. All of the experiments using animals were conducted under the supervision of, and with approval from the Animal Care and Use Committee of the Faculty of Agriculture, the University of Tokyo.

To determine the presence of viral RNA in bat samples, reverse transcriptase polymerase chain reaction (RT-PCR) was performed with a pair of consensus primers targeted to a highly conserved region of the RNA-dependent RNA polymerase (*RdRp*) gene [10]. Viral RNA was extracted from feces or intestinal tissue of the bats using a Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA). Complementary DNA was synthesized using a SuperScript III Kit (Invitrogen, Carlsbad, CA, USA). PCR was performed using GoTaq PCR Master Mix (Promega, Madison, WI, USA) and 0.2 μM primers (primer 1, 5′-GGTTGGGACTATCCTAAGTGTGA-3′; primer 2, 5′-CCATCATCAGATAGAATCATCATA-3′). PCR products were analyzed by agarose gel electrophoresis. Amplicons (440 nucleotides [nt] long) were gel–purified and directly sequenced with an ABI 3130 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

To detect anti-BtCoV antibodies in bat sera, western blot (WB) analysis was performed. Recombinant BtCoV nucleocapsid (N) protein produced by prokaryotic expression was used as an antigen. To amplify the full-length BtCoV N protein–coding region (1389 nt; AB543561), PCR was performed using KOD–Plus (Toyobo, Osaka, Japan) and 0.3 μM primers (forward primer, 5'-GAACAGATTGGAGGTATGTCTGGACGGAATAAGC-3'; reverse primer, 5'-TGCTCGAGTGCGGCCTTAGGATCTCTCATTAGCA-3'). The amplified fragment was cloned into the pE-SUMOstar vector (LifeSensors, Malvern, PA, USA). *E. coli* strain BL21 (DE3) was transformed with the recombinant plasmid. N protein expression was induced by the addition of 1 mM isopropyl–β–D–thiogalactopyranoside. The purity of proteins was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie brilliant blue (CBB) staining and WB analysis.

Recombinant BtCoV N protein was separated by 8 % SDS-PAGE. After blotting, nitrocellulose membranes were cut into strips and incubated for 1 h with bat serum samples (diluted 1:1000) or horseradish peroxidase (HRP)-

conjugated anti-6x His tag antibody (Abcam, Cambridge, UK) at a dilution of 1:5000. The strips were rinsed and further incubated for 1 h with rabbit anti-bat IgG antibody (diluted 1:1000) [15] and subsequently incubated for 1 h with HRP-conjugated donkey anti-rabbit IgG (diluted 1:2000; GE Healthcare, Milwaukee, WI, USA). Signals were detected using an Enhanced Chemiluminescence (ECL) Detection Kit (GE Healthcare).

From 2009 to 2011, we sampled a total of 179 bats at five sites in the Philippines. To detect viral RNA, RT-PCR was performed using a pair of primers for a conserved 440-nt region within the coronavirus *RdRp* gene. Intestinal samples containing feces obtained in 2009 and feces collected in 2010 and 2011 were examined. As shown in Table 1, the overall prevalence of BtCoV was 53/179 (29.6 %). Amplified fragments were sequenced directly for characterization. A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast) suggested that most of the sequences obtained in this study were similar to the sequence we reported previously (BtCoV/Philippines/Diliman1525G2/2008). These sequences were >98 % similar to one another and were obtained from lesser dog-faced fruit bats (*Cynopterus brachyotis*) and greater musky fruit bats (*Ptenochirus jagori*). Sequences from greater musky fruit bats (5/16 specimens positive by RT-PCR) that were similar to BtCoV/Philippines/Diliman1525G2/2008 were also found (87 % identical at the nucleotide level and 95 % identical in their translated amino acid sequences). Sequences that were >98 % identical were also amplified from Philippine sheath-tailed bats (*Emballonura alecto*), large rufous horseshoe bats (*Rhinolophus rufus*), Geoffroy's rousette bats (*Rousettus amplexicaudatus*), and lesser long-tongued fruit bats (*Macroglossus minimus*). A novel sequence was detected in a diadem roundleaf bat (*Hipposideros diadema*), which had 82 % similarity to BtCoV/A977/2005, found in China [16], and 75 % to the BtCoV we reported previously (BtCoV/Philippines/Diliman1552G1/2008) [14]. Phylogenetic reconstruction from isolated sequences was performed using MEGA5 software [17] (Fig. 1). This suggested that one of the detected sequences (from the diadem roundleaf bat) was related to members of the genus *Alphacoronavirus* (BtCoV2231/Philippines/2010), while most of the BtCoVs belonged to the genus *Betacoronavirus* (BtCoV/Philippines/Diliman1525G2/2008 and BtCoV2265/Philippines/2010). The two sequences (BtCoV2265/Philippines/2010 and BtCoV2231/Philippines/2010) were deposited into the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) with the accession numbers AB683970 and AB683971, respectively.
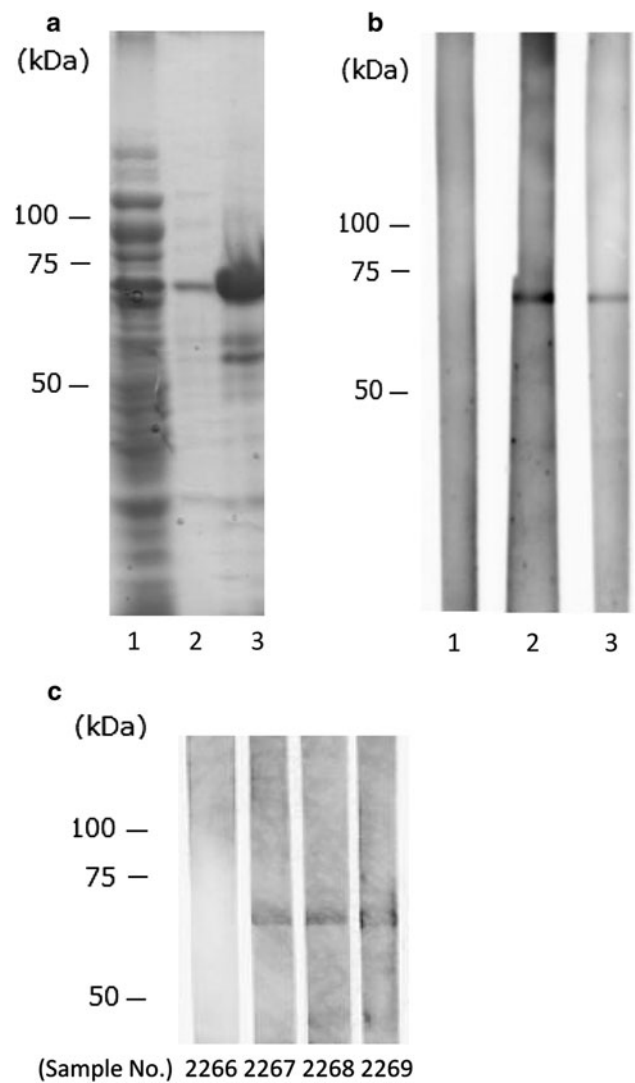
WB analysis was performed to detect antibodies to the CoV N protein. The 6x His-SUMO–fused full–length N protein was expressed in BL21 (DE3) cells and used as antigen. The expression of the ∼63-kDa recombinant
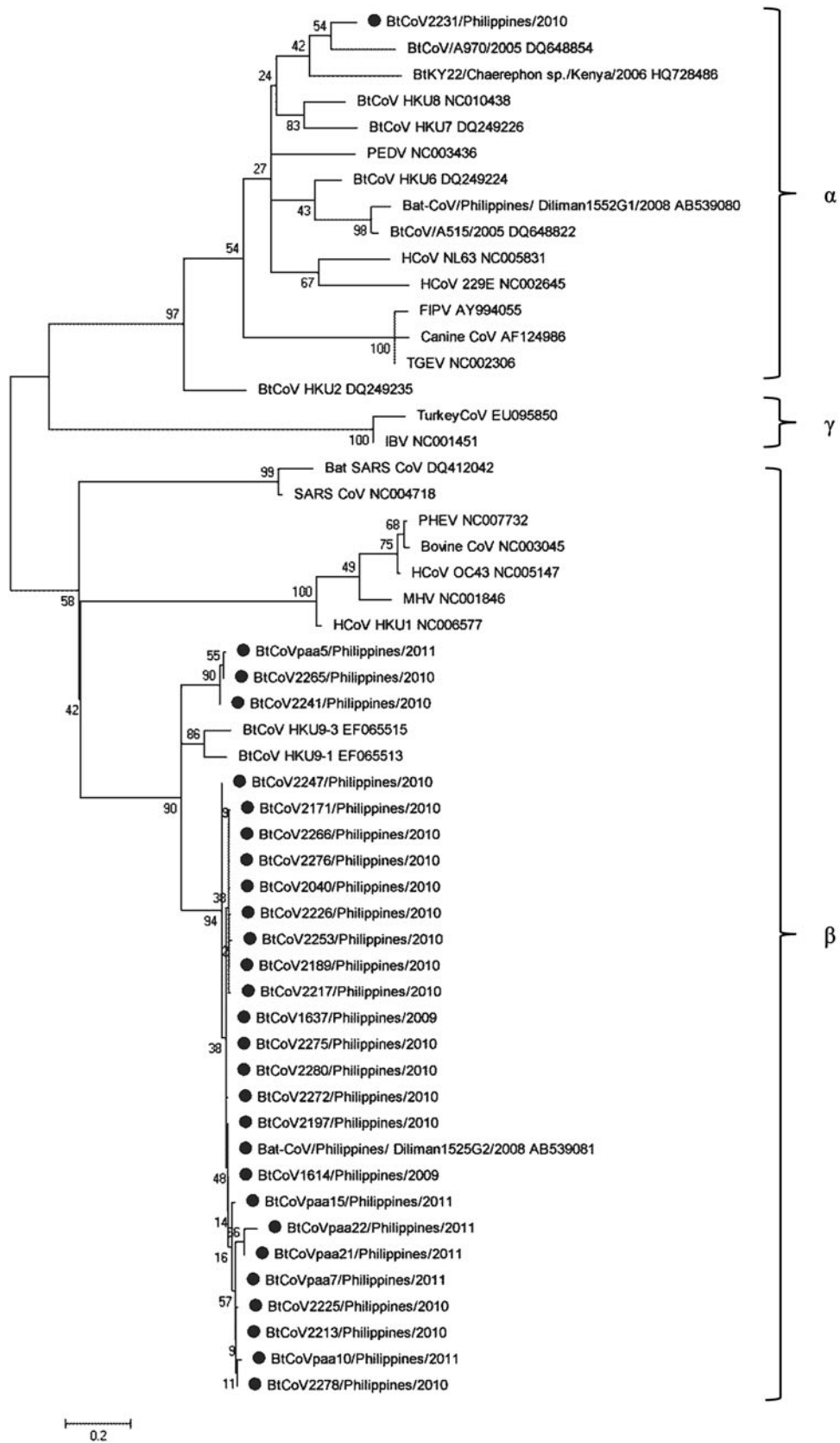
◄ **Fig. 1** **Phylogenetic analysis of isolated BtCoVs.** A phylogenetic tree was constructed by the maximum-likelihood method, using a conserved 372-nt sequence from the *RdRp* genes of isolates of BtCoV. Virus lineages detected in this study are marked with black circles. The percentage of replicate trees in which the associated taxa clustered in the bootstrap test (1000 replicates) is shown next to the branches. Phylogenetic analysis was performed using MEGA5 [17]. This model was selected by a modelfit using MEGA5 [17]. Coronaviral sequences used for comparison and their GenBank accession numbers were as follows: human coronavirus 229E (HCoV 229E; NC_002645), human coronavirus NL63 (HCoV NL63; NC_005831), human coronavirus OC43 (NC_005147), human coronavirus HKU1 (NC_006577), SARS coronavirus (NC_004718), canine coronavirus (AF_124986), feline infectious peritonitis virus (FIPV; AY_994055), transmissible gastroenteritis virus (TGEV; NC_002306), porcine epidemic diarrhea virus (PEDV; NC_003436), porcine hemagglutinating encephalomyelitis virus (PHEV; NC_007732), bovine coronavirus (NC_003045), murine hepatitis virus (MHV; NC_001846), avian infectious bronchitis virus (IBV; NC_001451), turkey coronavirus isolate MG10 (EU095850), BtCoV HKU2 (DQ249235), BtCoV HKU6 (DQ_249224), BtCoV HKU7 (DQ_249226), BtCoV HKU8 (NC_010438), BtCoV HKU9-1 (EF_065513), BtCoV HKU9-3 (EF_065515), bat SARS-CoV Rf1 (DQ_412042), BtCoV/A970/2005 (DQ_648854), BtCoV/A515/2005 (DQ_648822), BtKY22/Chaerephon sp./Kenya/2006 (HQ728486), BtCoV/Philippines/Dilliman1552G1/2008 (AB_539080) and BtCoV/Philippines/Dilliman/1525G2/2008 (AB_539081)

Fig. 2 **Expression and western blotting of the recombinant BtCoV N protein.** (**a**) CBB staining of the recombinant N protein. BL21 (DE3) cells expressing recombinant N protein were suspended in PBS containing 1 % Triton X-100, followed by sonication and centrifugation. Supernatants were collected (lane 1), and the pellet was resuspended in 0.5 % Triton X-100/PBS. After another round of centrifugation, the secondary supernatant was collected (lane 2). Two supernatant samples and the pellet (lane 3) were analyzed by SDS-PAGE. The gel was stained with CBB. (**b**) Western blotting of the recombinant N protein. Secondary supernatant (lane 2 in a) was separated by 8 % SDS-PAGE and detected by serum from a CoV-negative bat (lane 1 diluted 1:1000), serum from a BtCoV-infected bat (lane 2, diluted 1:1000) [14], and HRP-conjugated anti-6x His antibody (lane 3, diluted 1:5000). Strips incubated with bat sera were subsequently detected using rabbit anti-bat IgG antibody (diluted 1:1000) as the secondary antibody, and HRP-conjugated anti-rabbit IgG antibody (diluted 1:2000) as the tertiary antibody. (**c**) WB analysis of bat sera. Recombinant BtCoV N protein was detected by bat serum specimens as described in Fig. 2b

protein was confirmed by CBB staining and WB using an anti-6x His antibody (Fig. 2). As the recombinant N protein was present in the pellet after centrifugation, this pellet was resuspended in phosphate-buffered saline (PBS) containing 0.5 % Triton X-100. It was confirmed that the recombinant protein was pure and showed a single band in WB using an anti-6x His antibody (Fig. 2b). The secondary supernatant (isolated after two rounds of centrifugation of the *E. coli* cells) was used as antigen to detect antibodies to BtCoV. Bat serum samples that detected a 63-kDa band at a dilution of 1:1000 were considered positive (Fig. 2c). Sera from 10 Leschnault rousette bats (*Rousettus leschnaulti*), which were obtained from a zoo in Japan, were used as negative controls; none showed bands at 63 kDa. As a positive control, serum from coronavirus infected Leschnault rousette bat was used [14]. Table 1 displays the prevalence of anti-BtCoV antibodies; 119/179 (66.5 %) serum specimens had antibody against N protein. Total WB positivity correlated with total RT-PCR positivity ($p < 0.01$, Fisher's exact test).

Our surveys revealed that diverse populations of BtCoVs are continuously circulating in bats in the Philippines. Interestingly, CoVs in two different lineages were detected in greater musky fruit bats (BtCoV/Philippines/Diliman1525G2/2008 and BtCoV2265/Philippines/2010). Because this bat species is endemic to the Philippines [18], BtCoVs may have adapted to the local bat species and developed a unique ecology. While the bat-CoV/Philippines/Diliman1525G2/2008 lineage was only present in

lesser dog-faced fruit bats and greater musky fruit bats, the BtCoV2265/Philippines/2010 lineage was detected in various bat species, including both insectivorous and

frugivorous bats. These observations imply that interspecies jumping has occurred with BtCoVs in the Philippines. Given the wide host range of mammalian coronaviruses, it would not be surprising if BtCoVs crossed species barriers between frugivorous and insectivorous bats as well as barriers between different frugivorous bats. Besides SARS-CoV, cross-species transmission events have been implicated in the emergence of novel CoVs [19]. Recombination commonly occurs among CoVs [20] and also seems to happen in BtCoVs [21]. These two features could enable novel BtCoVs to emerge, leading to diversity and complexity among BtCoVs.

While numerous surveys to detect BtCoV RNA have been conducted, serological studies to find anti-CoV antibodies in bats are limited. It is possible that this lack of information has made our understanding of BtCoVs obscure. Only a few studies have investigated antibodies to SARS-related coronavirus in bats [4, 22, 23]. Our survey revealed that antibodies to BtCoV N protein were highly prevalent in bat species (66.5 %). This observation also supports the idea of continuous circulation of BtCoVs in the Philippines and implies that repetitive infection enables BtCoVs to circulate among bat colonies.

We previously reported two lineages of BtCoVs in the Philippines. One was an alphacoronavirus and the other was a betacoronavirus. In this study, we found two more distinct lineages: a betacoronavirus related to another previously described virus [14] and a novel alphacoronavirus. Our genetic and serological surveys suggest the widespread distribution and diversity of BtCoVs in the Philippines.

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