Detection of Coronavirus Genomes in Moluccan Naked-back Fruit Bats in Indonesia

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Abstract

Bats have been shown to serve as natural reservoirs for numerous emerging viruses including the severe acute respiratory syndrome coronavirus (SARS-CoV). In the present study, we report the discovery of bat CoV genes in Indonesian Moluccan naked-back fruit bats (*Dobsonia moluccensis*). The partial RNA-dependent RNA polymerase gene was detected in feces and tissues samples of the fruit bats and the helicase and a region between the partial RdRp and partial helicase genes could also be amplified from fecal samples. Phylogenetic analysis suggested the existence of these bat CoVs is related to the genus *Betacoronavirus*.

Keywords: Bat coronavirus, fruit bats, Indonesia

Coronaviruses (CoVs) are known to cause infections both in human and animals, with varying symptoms ranging from mild respiratory illness to severe infections resulting in death [1]. In addition to the respiratory tract, coronaviruses can also affect other organs in the body, including the gastrointestinal tract, liver, kidney, and brain of both humans and animals [2]. The pandemic of Severe Acute Respiratory Syndrome (SARS) in 2002 -2003 [3, 4] and the emergence of Middle East Respiratory Syndrome (MERS) [5] in 2012 are examples whereby human infections have resulted in significant mortality. In the case of SARS, the mortality rate reached up to 10% of cases resulting from respiratory failure [6-8].

Coronaviruses are the largest known RNA viruses with genomes up to 32 kb and belong to order *Nidovirales*, family *Coronaviridae*. These viruses possess an envelope and single-stranded positive-sense RNA [9]. Currently, four genera have been described: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [10]. *Alphacoronavirus* and *Betacoronavirus* are known to cause human disease whereas *Gammacoronavirus* and *Deltacoronavirus* are causative agents of animal disease [11]. Bats have been known as reservoir for many emerging infectious diseases that have a zoonotic potency [12]. Some of these bats were closely related to the origin of SARS-CoV [13] and MERS-CoV [14]. In Southeast Asian region, bats harboring coronaviruses have also been discovered in a number of countries, including the Philippines [15, 16] and Thailand [17]. However, there have been no studies focused on CoVs in Indonesia. Herein we report discovery of bat CoVs in Moluccan naked-backed fruit bats, *Dobsonia moluccensis,* in Indonesia.

We collected fruit bats samples from three regions in Indonesia: Paguyaman District, Gorontalo Province in 2012 (n = 37) and 2013 (n = 15); Surabaya District, East Java Province (n = 3) in 2012; and Yogyakarta District, Yogyakarta Special Province (n = 19) in 2012. All animal experiments were conducted in accordance with the ethical guidelines of the Animal Care and Use Committee of the Veterinary Teaching Hospital, Bogor Agricultural University. Fecal, tracheal swabs, and tissues samples including brain, lung, liver, spleen, and kidney from each bat were collected and placed into RNAlater (Life Technologies, Carlsbad, CA). All samples were exported with the permission of the Directorate General of Livestock and Animal Health Services, the Ministry of Agriculture, Republic of Indonesia to Japan.

Morphological features and nucleotide sequence analysis of both mitochondrial 16s rRNA and cytochrome *b* were conducted as described previously [18] to identify the species of each fruit bat. Feces and tissue samples were subjected to RNA extraction with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The RNAs were then screened for genomes of CoVs using a nested RT-PCR with primers sets which specifically amplify the conserved region of RdRp, as described previously [19] (A region in Fig. 1). The

helicase region was amplified using degenerate primers specific for the *Betacoronavirus* group [5] (B region in Fig. 1). PCR products of both RdRp and helicase regions were gel-purified and nucleotide sequencing was performed using a BigDye Terminator v.1.1/3.1 Cycle Sequencing Kit (Life Technologies) and analyzed with an ABI Prism 3130 Genetic Analyser (Life Technologies). OneStep RT-PCR was performed using new primer set and PrimeScript II High Fidelity OneStep RT-PCR Kit (TaKaRa, Tokyo, Japan) to amplify the region not covered in the first and second sets of PCR experiments (C region in Fig. 1). Following alignment of the sequences obtained in this study, the forward primer (5′-CACGCAACTTGTTGTAATGCGTCAGAGA-3′) and reverse primer (5′-CACGTGCTTTTGCAGGCACTATACGAC-3′) corresponded to the RdRp gene at nucleotide position 14850-14877 and to the helicase gene at nucleotide position 16714-16740, respectively, of bat coronavirus (BatCoV) HKU9 (NC009021). The obtained fragments covering partial RdRp and partial helicase genes (D region in Fig. 1) were subjected to nucleotide sequence analysis.

PCR-amplified nucleotide sequences were aligned and compared to the sequences of known CoVs using the MEGA 6 program. Nucleotide identity values were calculated using GENETYX software ver. 10 (GENETYX, Tokyo, Japan). The phylogenetic trees were constructed using maximum-likelihood tree method with Tamura-Nei model and 1000 bootstrap replicates [20, 21] based on nucleotide sequences.

Virus isolation was attempted by inoculation of African green monkey kidney (Vero E6), Yaeyama flying fox kidney (FBKT), and Leschenault's rousette kidney (DemKT1) cells. Vero E6 cells were obtained from Prof. Ayato Takada (Research Center for Zoonosis Control, Hokkaido University, Hokkaido, Japan) [22] and FBKT and DemKT1 cells were obtained from Prof. Ken Maeda (Yamaguchi University, Yamaguchi, Japan) [23]. Fecal and tissue homogenates which positive for CoV RNA were used for inoculation of cells after clearance by centrifugation at 1000 x *g* for 5 min. Cells were incubated at 37^{o}C for 30 min with fecal samples and for 1 h with tissue homogenates. Inocula were replaced by fresh culture media supplemented with 2% FBS, 2% antibiotic-antimycotic solution containing penicillin (10,000 U/ml), streptomycin (10 μg/ml) and amphotericin B (25 μg/ml) (Sigma, St. Louis, MO), and gentamycin (25 μg/ml). The supernatants of cultured cells were passaged to fresh cell monolayers each week. RNAs were extracted from culture supernatants and subjected to nested RT-PCR targeting the RdRp gene up until the fifth passage. The presence of cytopathic effect (CPE) was also checked daily until the end of the experiment.

The species of the captured bats were shown to be *Dobsonia moluccensis, Acerodon celebensis, Pteropus sp.* (genetically close to *Pteropus hypomelanus*), and *Pteropus vampyrus* (Table 1). Three samples from a total of 74 fecal samples from *Dobsonia* species produced a fragment of 455 bp in length after removal

of primer region by nested RT-PCR which was of the expected size of the RdRp fragment (A region in Fig. 1 and Table 1). The samples were IFB2012-8F, IFB2012-13F, and IFB2012-17F. The RdRp gene is the most common gene used for PCR amplification in CoV surveillance since it contains the most conserved regions of all CoVs [24]. Sequence analysis demonstrated that all samples contained the sequence of the RdRp gene conserved motif A (DYPKCD) and C (XSDD) which form the polymerase catalytic active site [24]. A 100% homology was found in the obtained partial RdRp sequence of IFB2012-13F and IFB2012-17F. There were 2 nucleotide substitutions when the RdRp sequences of IFB2012-13F and IFB2012-17F when compared with the RdRp sequence of IFB2012/8F.

In addition to the partial RdRp gene, a partial region of the helicase gene was also amplified producing a fragment of 355bp (B region in Fig. 1) from the three samples which were positive for PCR targeting the RdRp region. In this helicase gene, no nucleotide differences were found between IFB2012-13F and IFB2012-17F. However, substitutions in 2 nucleotides were observed between the IFB2012-8F and IFB2012-13F or IFB2012-17F.

After sequencing of both partial RdRp and partial helicase regions, we designed new primers to amplify sequences between these regions (C region in Fig. 1). A 1335-bp fragment was detected in 2 samples (IFB2012-8F and IFB2012-17F). A fragment (D region in Fig. 1), referred to as partial RdRp-helicase, were then constructed by incorporating overlapping sequence of partial RdRp, partial helicase, and a region in between. The nucleotide sequence comparison of partial RdRp-helicase from IFB2012-8F and IFB2012-17F showed 13 nucleotide substitutions. BLAST searches showed that IFB2012-8F and IFB2012-17F had the highest nucleotide identities with BatCoV HKU9-10-2 and BatCoV KY06 at 86% and 85%, respectively. Following removal of primer sequences from the obtained partial RdRp-helicase gene at the 5' and 3'end, the length of the sequence was 2,116 bp and a phylogenetic tree comparing the sequence of the samples with known CoVs, including SARS-CoV and MERS-CoV, was constructed (Fig. 2). The phylogenetic tree showed that IFB2012-8F and IFB2012-17F formed a distinct branch that was closely related to BatCoV HKU9 (NC009021), HKU9-2 (EF 605514), HKU9-5-2 (HM211099), and HKU9-10-2 (HM211101) from China [25] as well as BatCoV KY06 from Kenya (HQ728483) [26]. All of these CoVs belong to the genus *Betacoronavirus*.

Nucleotide sequences of partial RdRp-helicase gene derived from fecal samples were deposited under accession number AB918718 to AB918719 in DNA Data Bank of Japan (DDBJ, Tokyo) nucleotide database from IFB2012-17F and IFB2012-8F, respectively.

Various tissues and tracheal swab samples collected from the bats where the feces showed a positive result were also subjected to nested RT-PCR for the partial RdRp region. Spleen tissue of IFB2012-13F, brain tissue of IFB2012-17F, and lung tissue of IFB2012-17F showed positive signals for RdRp gene amplification. The sequence analysis of tissues samples showed sequences identical with the partial RdRp sequence obtained from feces samples (IFB2012-13F and IFB2012-17F). Respiratory epithelium is known as a target for CoVs [27] and therefore detection of bat CoV genome fragments in lung samples might be expected. However, the detection of CoV genome fragments in spleen and brain samples suggested that bat CoV infection in these species may not be restricted to the respiratory or alimentary tracts.

Virus isolation attempted using Vero E6, FBKT, and DemKT1 cells was unsuccessful. No cytopathic effects (CPEs) was observed during cell cultures and viral RNA was not detected by RT-PCR from the culture supernatants after the fifth passage.

Nucleotide sequencing and phylogenetic analysis of partial RdRp-helicase regions of the samples in the present study and these of known CoVs suggest that obtained CoVs genome fragments in this study is related to the genus *Betacoronavirus*. More than 90% identity of amino acid sequence in the conserved replicase domain: ADRP, nsp5 (3CLpro), nsp12 (RdRp), nsp13 (helicase), nsp14 (ExoN), nsp15 (NendoU), and nsp16 (O-MT) should be observed in order to define that one CoV species belongs to the same species of other CoVs [28]. Therefore, CoVs detected in this study could not be defined in the species level with regards only certain regions of conserved replicase domains were determined. However, the number of nucleotide substitutions over the partial RdRp-helicase sequence between IFB2012-8F and IFB2012-17F and phylogenetic analysis of partial RdRp-helicase suggested that the fruit bats harbor variants of a single coronavirus strain.

In this study, CoV genomes located within the Betacoronavirus genus were detected in *Dobsonia moluccensis*, known as Moluccan naked-backed fruit bat in Indonesia. *Dobsonia moluccensis* belongs to family *Pteropodidae*, which is known to harbor a number of viruses including CoVs [25, 29], Paramyxoviruses [18, 30] and Rhabdoviruses [29, 31]. Nipah and Hendra viruses were also detected in *Dobsonia moluccensis* [32, 33].

This report highlights that bats are a natural reservoir for various viruses in Indonesian fruit bats and be zoonotically transmitted, since fruit bats are also consumed in certain parts of Indonesia.

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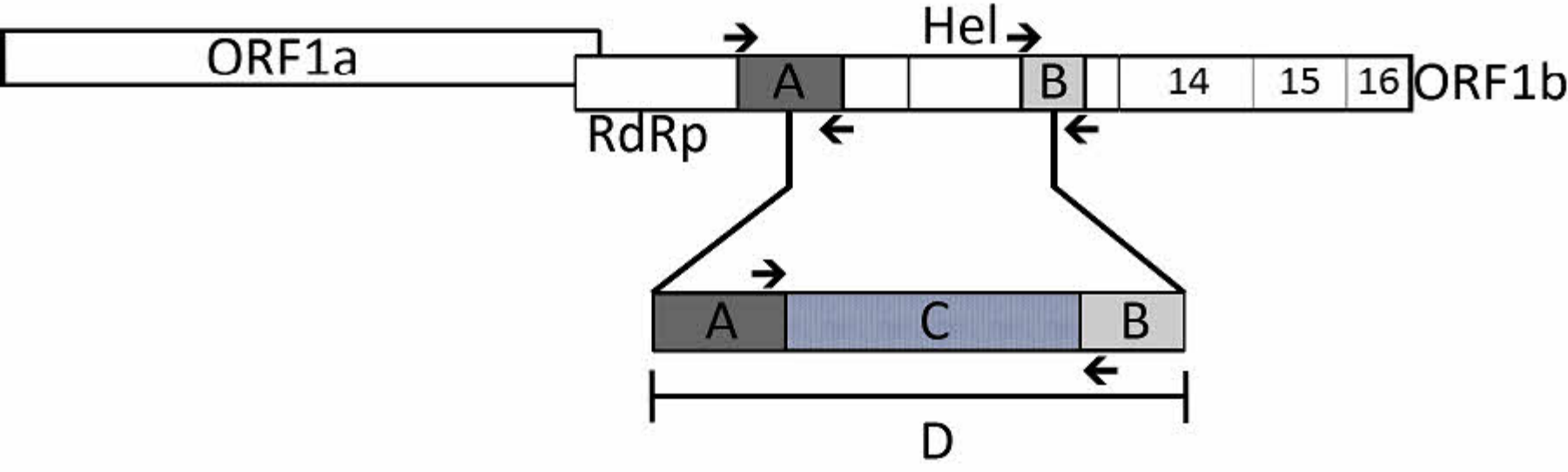
Figure legends

Fig 1. Schematic diagram of amplified genes for detection of CoVs from fruit bats based on coronavirus replicase gene. ORF1a and ORF 1b are constituents of replicase gene. ORF1a, open reading frame 1a; RdRp, RNA-dependent RNA polymerase; Hel, helicase; ORF1b, open reading frame 1b; A, partial RdRp gene (455 bp); B, partial helicase gene (355 bp); C, region between amplified partial RdRp and partial helicase gene (1,335 bp); D, partial RdRp-helicase gene (2,145 bp); arrows, primers' direction used in RT-PCR.

Fig 2. Phylogenetic tree of partial RdRp-helicase using a 2,116 bp nucleotide sequence following removal of primer sequences at the 5' and 3' end of the partial RdRp-helicase gene obtained from fruit bats samples (IFB2012-8F and IFB2012-17F) and various coronaviruses derived from GenBank. The tree was constructed using Maximum Likelihood method by MEGA 6 software. Bootstrap values were calculated on 1000 replicates and are shown next to the branches. Bat coronaviruses detected in this study are shown with black square mark. SARS-CoV, severe acute respiratory syndrome coronavirus; Avian IBV, avian infectious bronchitis virus; CoV, coronavirus.


ORF1a
Hel
A
B
14
15
16
ORF1b
RdRp
A
C
B
D

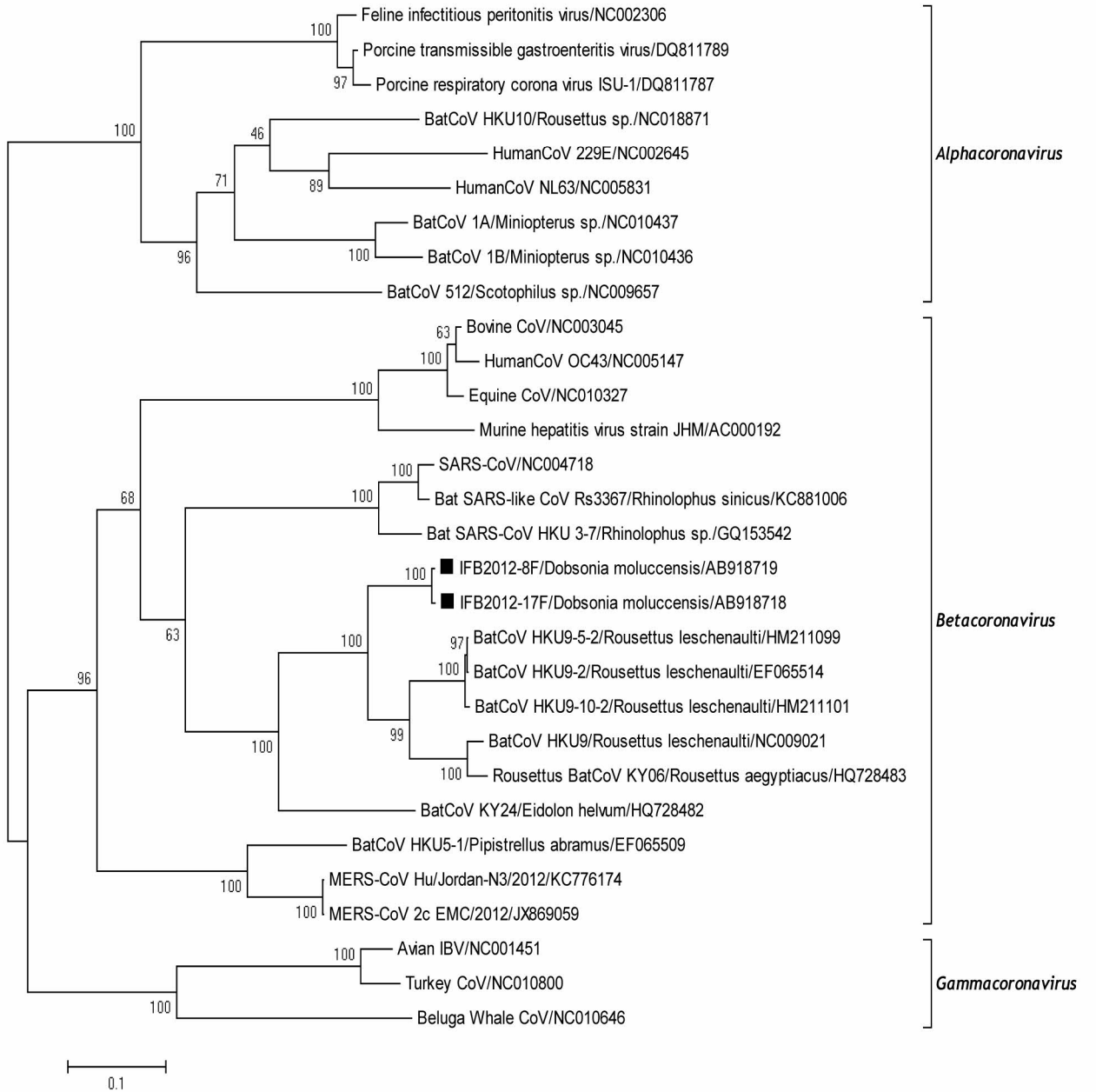

Feline infectitious peritonitis virus/NC002306
Porcine transmissible gastroenteritis virus/DQ811789
Porcine respiratory corona virus ISU-1/DQ811787
BatCoV HKU10/Rousettus sp./NC018871
HumanCoV 229E/NC002645
HumanCoV NL63/NC005831
BatCoV 1A/Miniopterus sp./NC010437
BatCoV 1B/Miniopterus sp./NC010436
BatCoV 512/Scotophilus sp./NC009657
Alphacoronavirus
Bovine CoV/NC003045
HumanCoV OC43/NC005147
Equine CoV/NC010327
Murine hepatitis virus strain JHM/AC000192
SARS-CoV/NC004718
Bat SARS-like CoV Rs3367/Rhinolophus sinicus/KC881006
Bat SARS-CoV HKU 3-7/Rhinolophus sp./GQ153542
IFB2012-8F/Dobsonia moluccensis/AB918719
IFB2012-17F/Dobsonia moluccensis/AB918718
BatCoV HKU9-5-2/Rousettus leschenaulti/HM211099
BatCoV HKU9-2/Rousettus leschenaulti/EF065514
BatCoV HKU9-10-2/Rousettus leschenaulti/HM211101
BatCoV HKU9/Rousettus leschenaulti/NC009021
Rousettus BatCoV KY06/Rousettus aegyptiacus/HQ728483
BatCoV KY24/Eidolon helvum/HQ728482
BatCoV HKU5-1/Pipistrellus abramus/EF065509
MERS-CoV Hu/Jordan-N3/2012/KC776174
MERS-CoV 2c EMC/2012/JX869059
Betacoronavirus
Avian IBV/NC001451
Turkey CoV/NC010800
Beluga Whale CoV/NC010646
Gammacoronavirus
0.1

Table 1 Detection of Coronaviruses in fruit bats targeting RdRp of fecal samples by nested RT-PCR.

Year	Bat Species	Location	No. samples tested	No. positive samples
2012	*Dobsonia mollucensis*	Paguyaman	17	3
	Acerodon celebensis		18	0
	Pteropus sp.		2	0
	Pteropus vampyrus	Surabaya	3	0
	Pteropus vampyrus	Yogyakarta	19	0
2013	*Acerodon celebensis*	Paguyaman	7	0
	Pteropus vampyrus		8	0
	Total		74	3