Bat Coronavirus of Pteropus alecto from Gorontalo Province, Indonesia

by Triltje Ransalele 7

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Bat Coronavirus of *Pteropus alecto* from Gorontalo Province, Indonesia

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Abstract

Bats have been known as natural reservoirs for potential emerging infectious viruses, such as Lyssaviruses, Coronaviruses, Ebola viruses, Nipah virus, and many others. Because of their abudance in population, wide distribution and mobility, bats have a greater risk as source for zoonotic transmission than other animals. Despite the facts of their role as reservoirs for many pathogens, not until an epidemic of Severe Acute Respiratory Coronavirus (SARS-CoV) in 2003 and Middle-East Respiratory Syndrome Coronavirus (MERS-CoV) in 2012, that people pay much attention about coronavirus in bats. SARS-like virus also found in bats with a higher prevalence rate. This study aims to detect the coronavirus of bats in Gorontalo province Indonesia, characterization at the molecular level of the coronavirus genome and determining the level of kinship (through trees filogenetic). This study was conducted as part of bigger PREDICT Indonesia project, in particular to examine coronavirus in bats from Gorontalo province, Indonesia. As many as 95 rectal swab samples collected from flying foxes (*Pteropus alecto*) were analyzed in the laboratory using Consensus Polymerase Chain Reaction (PCR) technique to amplify the target sequence from RNA-dependent RNA Polymerase (RdRp) gene with 434 basepair product, resulted 24 samples determined as presumptive positive. Eight out of 24 presumptive positive samples by PCR were analyzed further by nucleotide sequencing and confirmed coronavirus positive. Phylogenetic tree analyses to the eight coronavirus confirmed-sequences were constructed with MEGA-6.0 . The conclusion was 24 out of 95 samples suggested as presumptive positive to Bat CoV. Eight out of 24 samples were analyzed further by nucleotide sequencing and have similarities in the kinship. Three samples had the 98% nucleotide identity to BatCoV from Indonesia and five samples were 85-88% nucleotide identity to BatCoV from Thailand.

Keywords: Consensus PCR, Coronavirus, Indonesia, Pteropus alecto

Background

Indonesia has a megabiodiversity, both flora and fauna, and it has about 162 million hectares of forest area with a variety of ecosystems, including the habitat of bats. Sulawesi Island, which most of their area has included in the Wallace line ecological zone, become the second largest home to flora or fauna next to Brazil in terms of biodiversity (Achmaliadi *et al*., 2011). About 62 species of bats in the world are found in Sulawesi (Heinrichs *et al*., 1997). Bats are known as potential reservoir for emerging pathogens. Because of their abundance, distribution, and mobility, bats give high risk for transmission to other

animals (Calisher *et al*., 2006). Human interaction with wildlife is mediated by multiple triggers (drivers): forest clearing, mining industry, eco-tourism, transportation, wildlife hunting and illegal trading, as well as consumption of wildlife meat. Sulawesi an community (Minahasa) known as consumer of domestic animals or wildlife. Bats are one of the most favorite meat, even it becomes popular extreme culinary (Whitten *et al*., 1987)

Previously, the infection of coronavirus (CoV) in human received less attention until a new type of coronavirus causing epidemics in humans was reported; This Severe Acute Respiratory Syndrome Coronavirus was discovered in 2003 and the Middle-East Respiratory Syndrome Coronavirus (Mers-CoV) in 2012 which infected more than 8000 people in the world and caused 774 deaths (Poon *et al*.,2004). Common symptoms of SARS include a high fever over 38°C of body temperature, followed by symptoms of pneumonia (CDC, 2015;Mahon *et al*.,2014). In Southeast Asia, it was documented that bats are known to carry coronavirus in several countries, including the Philippines (Watanabe *et al*.,2010) and Thailand (Gouilh *et al*.,2011).

Surveillance of virus in bats indicates the type of virus which is classified as pathogens that causing emerging infectious diseases and potentially zoonotic. The interaction between bats and humans (human-animal interface) especially in North Sulawesi region could increase the risk of transmission of pathogen against zoonotic diseases. Only a few studies focused on coronavirus of bats were conducted in Indonesia. Therefore, it is necessary to conduct research on identification and characterization of CoV in bats from their natural habitats in Indonesia, for example Gorontalo Province.

Materials and Methods

This project was conducted under research grant from PREDICT Indonesia in the EPT-1 Program.

Sample collection

We obtained 95 bats of one species: black flying fox (*Pteropus alecto*) during 2013 field sampling from Olibuu mangroves, Gorontalo province, after receiving permission from the goverment. All bats captured from mangrove forests Olibuu using relevant equipment, conditioned on a certain place and then taxonomically classified on the basis of morphology. The specimens were sampled from urine or uro-genital swab, blood, fecal or rectal swab, throat swab, and skin biopsy when necessary for the host genetic identification. All specimens in lysis buffer or VTM (Viral Transport Medium) were collected in accordance with the Guidelines for the Institutional Animal Care and USE Committee at the PRC-IPB with protocol number approval PRC-16-D001 and stored in a cold chain in order to keep the low temperature. Recommendations to carry out the study was issued by Indonesian Institute of Science (LIPI) approval number: 1689/IPH.1/HK.04.04/VII/2013. Approval for shipping of specimens from Olibuu mangrove forests to Bogor was issued by the Directorate General of Conservation of Natural Resources and Ecosystem, Ministry of Forestry, number 02/SATS/BKSDA-28/2013

RNA Extraction and cDNA synthetis

Viral RNA was extracted from rectal swab specimens using QIAamp viral RNA minikit (QIAGEN, Hilden, Germany) according to the manufacture's instructions. These RNA then used as the template for reverse transcription using Superscript ™ III First-Strand cDNA Synthesis System for RT-PCR (Life Technologies, CA, USA). Briefly, 10 μL of extracted RNA containing 1 μL of 10 mM dNTPs, 1 μL of 50 pmol/μL random hexamer was incubated at 65ºC for 5 minutes. The mixture then added to 10 μL of a reverse transcriptase enzyme mix containing 10 RT buffer, 25mM $MgCl_2$, mM MgSO4, 0.1 M DTT, RNAs out, and 1 μL (200U) of reverse transcriptase enzyme. Samples were subjected to an initial cycle of 50°C for 50 min and 85°C for 5 min. One

microliter of RNaseH 2U/ μL was added and incubated at 37ºC for 20 min

PCR and DNA sequencing

All cDNA samples obtained from Olibuu mangroves were tested using conventional nested PCR. We used two sets of consensus primers amplifying highly conserved region of the RNA-dependent RNA polymerase (RdRp) gene, modified by Watanabe *et al*., 2010. As much as 2.5 μL of cDNA was added to a 22.5 μL reaction mixture of 2x KAPA Taq Hotstart ready mix (containing 1U KAPA Taq DNA polymerase, KAPA Taq buffer, 0.2 mM dNTPs, 1.5mM $MgCl_2$) (KAPA Biosystem, Boston, USA), 1 μL of each primer (10 pmol/ μL) and nuclease free water. The primer for first round PCR were CoV-Forward-1: GGTTGGGAYTAYCCHAA-RTGTGA and CoV-Reverse-1: CCATCA-TCASWYRAATCATCATA; and for the second round were CoV-forward-2 as forward primer: GAYTAYCCHAARTGT-GAUMGWGC and the same CoV-Reverse-1 primer as used in first round. PCR amplification was undertaken in Thermal Cycler (Veriti Applied Biosystem) at 94 ºC for 2 minutes, 35 cycles at 94ºC for 20 sesonds, 50ºC for 30 seconds and 72ºC for 30 seconds and a final extension of 72ºC for 5 minutes. This protocols are the same for second round PCR. The amplified PCR product then analyzed by 1.8% agarose gel electrophoresis in tris-acetic EDTA (TAE buffer) and visualized in GelDoc machine (BioRad) using Quantity One program

Sequences and Data Analysis

The presumptive PCR positive samples were results then nucleotide sequenced in commercial sequencing provider. The results then analyzed by BioEdit program and further analyzed by alignment method using the Basic Local Alignment Search Tool (BLAST) program from NCBI. Sequences were aligned with previously published CoV sequences from GenBank by using ClustalW program. The GenBank accession numbers of all sequences used are noted in the taxon names in Figures 2. The partial RdRp gene sequences were trimmed to equal length about 390 bp. Phylogenetic trees were constructed to see the kinship of the virus using the Molecular Evolutionary Genetics Analysis 6.0 (MEGA 6.0) with neighbor-joining (NJ) method. Bootstrapping was perfomed to asses the robustness of tree topologies by using 1000 replicate (Tamura *et al*.,2013).

Results and Discussion

As many as 95 *Pteropus alecto* bats rectal swab samples were collected from Olibuu Mangrove Gorontalo Province Indonesia. Reverse transcribed-consensus nested PCR of 434 bp partial RdRp gene (Figure 1) were performed in this research and resulted 24 out of 95 samples (23.3%) as presumptive positive to CoV. Eight out of 24 presumptive positive samples by PCR were analyzed further by nucleotide sequencing and confirmed CoV positive.

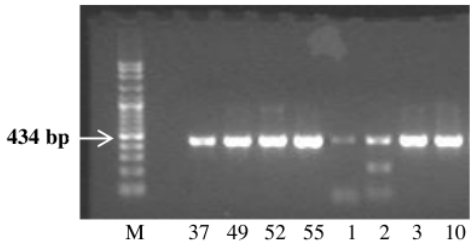

Figure 1. Visualization of nested PCR results of 8 rectal swab bats samples from Olibuu mangroves using CoV F4/Bat primers and CoV RVS3. M: 100 bp DNA ladder (Vivantis), lane 3-10: 434 bp of nested PCR positive results

Based on BLAST analysis results showed that the samples of INDSWBT101, INDSWBT102, and INDSWBT103 were 98% of nucleotide identity to BatCoV IFB2012-8F (AB918719) isolated from Indonesia *Dobsonia moluccensis* (Anindita, 2015). Meanwhile, samples of INDSWB-T180, INDSWBT195, INDSWBT192, INDSWBT110, INDSWBT198 were 85-88% nucleotide identity to BatCoV/b5576-2/S.hea/CB/Tha/6/2012 (KJ020607) isolated

from Thailand's *Scotophilus heathii* [12] showed in Table 1.

Table 1: Nucleotide identity of bat CoV from Olibuu *Pteropus alecto* rectal swab samples based on BLAST analysis

No	ID Number	Origin	% Virus Identity
1	INDSWBT 110	Olibuu Mangrove	85% Bat Coronavirus Thailand BtCoV/B55762/S.hea/CB/Tha/6/2012
2	INDSWBT 192	Olibuu Mangrove	85% Bat Coronavirus Thailand BtCoV/B55762/S.hea/CB/Tha/6/2012
3	INDSWBT 198	Olibuu Mangrove	85% Bat Coronavirus Thailand BtCoV/B55762/S.hea/CB/Tha/6/2012
4	INDSWBT 180	Olibuu Mangrove	88% Bat Coronavirus Thailand BtCoV/B55762/S.hea/CB/Tha/6/2012
5	INDSWBT 195	Olibuu Mangrove	88% Bat Coronavirus Thailand BtCoV/B55762/S.hea/CB/Tha/6/2012
6	INDSWBT 101	Olibuu Mangrove	98% Bat Coronavirus Indonesia IFB 2012-8F *Dobsonia moluccensis*
7	INDSWBT 102	Olibuu Mangrove	98% Bat Coronavirus Indonesia IFB 2012-8F *Dobsonia moluccensis*
8	INDSWBT 103	Olibuu Mangrove	98% Bat Coronavirus Indonesia IFB 2012-8F *Dobsonia moluccensis*

Based on Table 1 shows that Coronavirus (CoV) found in samples of bat originating from Olibuu Gorontalo province has similarities in nucleotide sequences with virus samples that have been found in Indonesia before by Anindita, 2015. She mentioned the coronavirus found in *Dobsonia moluccensis* bat species obtained from 3 regions in Indonesia, Paguyaman, Gorontalo, Surabaya and Jogjakarta. whereas the similarity of the sequences of the next nucleotides is 85-88% found in Thailand in the bat species *Scotophilus heathii* (Wacharapluesadee, 2015). Three samples had the 98% nucleotide identity to BatCoV from Indonesia and five samples were 85-88% nucleotide identity to BatCoV from Thailand

Phylogenetic analysis of nucleotide sequences of 390 bp of RdRp gene showed that 8 samples were clustered as Betacoronavirus genus and branched to two clustered groups. The first group consist of three specimens (INDSWBT101, IND-WBT 102, and INDSWBT103) were 99% nucleotide identity with each other were closely related and clustered to BatCoV IFB2012-8F from Indonesia (AB918719) and clustered with bat coronavirus BtKY78 from Kenya (GU065422), Bat CoV from Lebanon (KT368821), Bat CoV isolate Rousettus/Egypt/NRC-HKU-308 from Egypt (KT346242.1), Bat CoV 2265/Philippines/2010 from Philippines (AB683970), Rousettus Bat Alphacoronavirus strain ML_32I (KP895524), and Bat CoV (KU182986) from China. The second group consist of five specimens (INDSWBT180, INDSWBT195, INDSWBT192, INDSWBT110, and INDSWBT198) were 84% nucleotide identity among each other, therefore suggested these isolates were novel although were closely related to Bat CoV from Thailand (KJ020607) at the 85-88% identities.

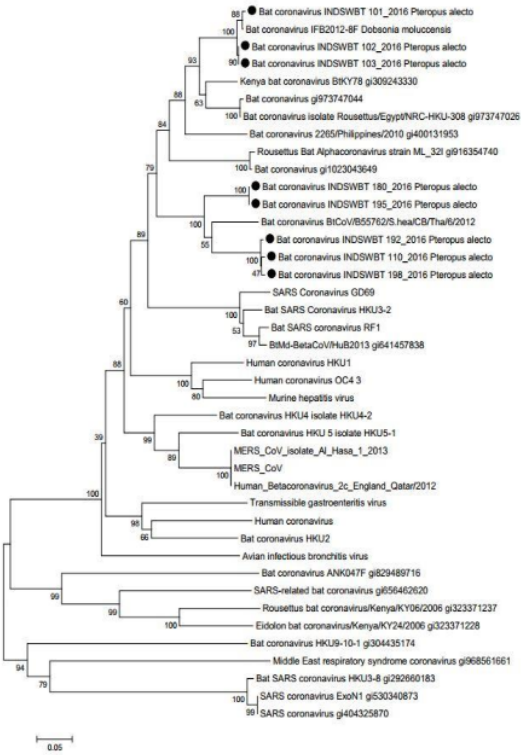

Figure 2: Phylogenetic tree of bat coronavirus based on RdRP partial gene constructed using Neighbor Joining method with 1000 times bootstrap. Values on each branch of the tree indicate the bootstrap values.

In this study, we found that 23.3% presumptive positive to bat coronavirus were detected from *Pteropus alecto* rectal swab specimens. Meanwhile, only 8

presumptive positive out of 97 samples (8.3%) were detected from throat swab specimens of those samples (unpublish data). This finding suggests that bat CoV circulation is found more around the digestive tract. This means to detect the genetic material of bats CoV is more likely found in rectal swab than throat swab, although most of CoV are transmitted by both and by aerosols of respiratory secretions. These data are consistent with the result of bat CoV detection from fecal sample or rectal swab or intestine tissue that previously reported. (Watanabe *et al*.,2010; Anindita *et al*.,2015; Wacharapluesadee *et al*.,2015). The species of bats captured from Olibuu Mangrove was identified as *Pteropus alecto* (black flying fox), meanwhile the CoV genetic material were detected closely related to the bat CoV found in Indonesian *Dobsonia moluccensis* (Moluccan naked-backed fruit bat) and Thailand's *Scotophilus heathii* (greater Asiatic yellow bat) (Anindita *et al*.,2015; Wacharapluesadee *et al*.,2015). Therefore, bat CoV with similar genetic material were found in different bats species and not solely correlated with one host species. This data also supported the previous study about the diversity of CoV detected in different bats species (Wacharapluesadee *et al*.,2015).

The RdRp gene is the one most commonly used for PCR amplification in CoV surveillance, since it contains the most region that are conserved in all CoV (Xu *et al*.,2003). The primers designed in this research were nested consensus primer that amplified the RdRp partial genes resulted about 434 bp PCR product modified of Watanabe et al 2010 (Anthony *et al*.,2013). The gene target position was a different region slightly more upstream. On the Human CoV genome (Strain 229E) it targets roughly nucleotides at the position 14,370-14,750 of CoV whole genome. The primers developed by modified some degenerate sequences to increase the ability of the assay to detect widely variant CoV in family level. A hemi-nested PCR step has also been conducted to increase the sensitivity. This step can be performed using a forward primer that is optimized for bat CoV, or other coronaviruses, depending on the sample being investigated (Anthony *et al*.,2013)

The BLAST and the phylogenetic tree result to the 8 bats CoV positive samples indicated there were two group bats CoV detected from Olibuu *Pteropus alecto*. Three samples (INDSWBT101, INDSWBT 102, and INDSWBT103) clustered with the known bat CoV that previously reported from Paguyaman, Indonesia with 98% nucleotide identity. Olibuu and Paguyaman are the same province region of Gorontalo Indonesia. Five specimens (INDSWBT180, INDSWBT195, INDSWBT192, INDSWB-T110, and INDSWBT198) possibly assumed as new bats CoV due to low similarity (85%) to others bat CoV in Genbank, although still clustered with bat CoV from Thailand. Further characterization to the whole RdRp gene or the other gene region of these bat CoV is needed to prove this assumption. In general, CoV in bats that taken from Olibuu mangroves, Gorontalo province were genetically still has a kinship with the others CoV found in some areas of the world (Kenya, China, Thailand, Philippine, Middle East, and Madagascar).

This study shows that fruit bats from Indonesia is a reservoir for viruses that have potentially zoonotic, because the fruit bats are also consumed in some areas in Indonesia. Although CoV does not cause illness in bats and the kinship of the findings of phylogenetic in this study were far distance with SARS and MERS-CoV, but this studies finding need more attention about the impact of the CoV to human health and could serve as an early warning. The information could serve as additional data to support global monitoring system for the emergence of bats CoV which potentially become dangerous pathogens to human health, thus further and assist the authorities to develop relevant policies. The threat of emerging pandemic diseases is facilitated by the interaction of wildlife and humans at human-animal interfaces. In Indonesia, as in other countries, increasing contact between wildlife and humans leads

to greater risk of human exposure to pathogens, both well-known and new. Habitat disturbance, such as forest clearing for agriculture or development purposes; transportation; handling of wildlife; and wildlife consumption, among other human-mediated practices, promotes direct opportunities for pathogens to move from wildlife to people. Development of early warning systems, including expanded surveillance and diagnostic capacity for potential disease threats to human beings, is urgently needed by government agencies, research, and academic institutions in order to better serve and protect the public

Conclusion

Twenty four out of 95 samples suggested as presumptive positive to Bat CoV. Eight out of 24 samples were analyzed further by nucleotide sequencing and confirmed as Bat CoV. Three samples had the 98% nucleotide identity to BatCoV from Indonesia and five samples were 85-88% nucleotide identity to BatCoV from Thailand.

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