Molecular detection of bat coronaviruses in three bat species in Indonesia

Ni Luh Putu Indi Dharmayanti, Diana Nurjanah, Harimurti Nuradji, Ibnu Maryanto, Indra Exploitasia, Risa Indriani

ABSTRACT

Bats are an important reservoir of several zoonotic diseases. However, the circulation of bat coronaviruses (BatCoV) in live animal markets in Indonesia has not been reported. Genetic characterization of BatCoV was performed by sequencing partial RdRp genes. Real-time polymerase chain reaction based on nucleocapsid protein (N) gene and Enzyme-linked immunosorbent assay against the N protein were conducted to detect the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral RNA and antibody, respectively. We identified the presence of BatCoV on Cynopterus brachyotis, Macroglossus minimus, and Rousettus amplexicaudatus. The results showed that the BatCoV included in this study are from an unclassified coronavirus group. Notably, SARS-CoV-2 viral RNA and antibodies were not detected in the sampled bats.

INTRODUCTION

Bats have important roles as natural reservoir hosts of zoonotic diseases and act as agents of zoonosis transmission, including the transmission of coronaviruses that led to severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) [1]. Bats have good adaptability skills, which support their resistance against several viral infections [2]. Studies in Indonesia have revealed that bats from several regions harbor Hendra and Nipah viruses [3,4]. There are more than 1,200 species of bats, and approximately 20% (239 species) have been reported in Indonesia [5-7]. This high diversity of bat species provides a wide variety of cell types and receptors that may facilitate the bat species becoming a potential source for coronavirus transmission [8].

Cross-species transmission of a virus from bats to humans can occur directly or indirectly through an intermediate host. The increasing number of zoonotic disease outbreaks may be due to human invasion of the natural habitat of bats and to hunting bats for food consumption [9,10]. Various interferences may cause the bats to experience stress, weakening their immune systems and potentially increasing the replication and shedding
This research was funded by the Indonesian Research Center for Veterinary Sciences Project Research 2019–2021.

The authors declare no conflicts of interest.

Conceptualization: Dharmayanti NLPI; Data curation: Nurjanah D, Maryanto I, Exploitasia I; Formal analysis: Dharmayanti NLPI, Nurjanah D; Funding acquisition: Dharmayanti NLPI; Investigation: Nurjanah D, Maryanto I; Methodology: Nurjanah D, Nuradji H, Maryanto I, Indriani R; Project administration: Dharmayanti NLPI; Resources: Dharmayanti NLPI; Software: Dharmayanti NLPI; Supervision: Dharmayanti NLPI; Validation: Exploitasia I, Indriani R; Visualization: Nuradji H, Maryanto I, Exploitasia I; Writing - original draft: Dharmayanti NLPI, Nurjanah D, Nuradji H, Maryanto I, Exploitasia I; Writing - review & editing: Dharmayanti NLPI, Nurjanah D, Nuradji H, Maryanto I, Exploitasia I.

Wild animal meat (“bushmeat”) comes from various sources, including bats. Transmission of bushmeat-associated pathogen occurs through direct contact with the body fluids and feces of the wild animals. Ebola virus, human immunodeficiency virus-1, monkeypox virus, and SARS-CoV are infectious pathogens associated with bushmeat [12-14].

SARS-CoV and MERS-CoV are zoonotic coronaviruses that believed originating from bats and have been transmitted to other species (civets and camels, respectively), which act as intermediate hosts before being transmitted to humans [15-17]. The SARS-CoV-2, which recently caused a worldwide pandemic, was shown to have a nucleotide similarity of up to 96.1% with BatCoV RaTG13 originating from Rhinolophus affinis and first isolated in 2013 [18,19].

Various types of interactions between bats, other animals, and humans may contribute to the interspecies transmission of coronaviruses, including interactions that may occur in live animal markets [15,20,21]. Live animal markets are located in most regions of Indonesia, 2 of which are the Tomohon market in Manado, North-Sulawesi and the Depok market in Surakarta, Central Java, where people sell wild animals for consumption and for other purposes [22]. Live animal markets are potential meeting points for the interspecies transmission of several diseases from animals to human or vice versa (i.e., zoonotic diseases) [12-14,23].

In 2015, Anindita et al. [24] reported a bat betacoronavirus (Bat betaCoV) in Dobsonia moluccensis acquired in Paguyaman, Gorontalo Province, Indonesia. Furthermore, Febriani et al. [25] reported that Pteropus alecto in Gorontalo Province carried Bat betaCoV. This study reports for the first time the presence of bat coronaviruses (BatCoV) in 3 species of bats sold by traders at live animal markets in Central Java Province, and bat collectors in West Java Province and Yogyakarta Province, Indonesia. The results of this study are expected to contribute to elucidating BatCoV ecology in Indonesia and identify the species of bats that are potential hosts of BatCoV.

MATERIALS AND METHODS

Sample collection
This animal-based experiment was approved by the Indonesian Agency for Agricultural Research and Development (IAARD), Institutional Animal Care and Use Committee (IACUC) under registration numbers Balitbangtan/BB litvet/M/01/2020 and Balitbangtan/BB litvet/M/01/2021. A total of 182 bats (126 samples from 2020 and 56 samples from 2021) were obtained from traders at animal markets and bat collectors at several cities or regencies in Central Java Province (Surakarta City and Magelang Regency), Yogyakarta Province, and West Java Province (Bogor City and Cianjur Regency). The collected samples were identified as Cynopterus brachyotis (n = 45), Macroglossus minimus (n = 5), Rousettus aplexicaudatus (n = 96) and Pteropus vampyrus (n = 36). Specimens for identification were collected from rectal swabs and blood sera. Rectal swab samples were placed in transport medium (Dulbecco’s modified Eagle’s medium; GIBCO, Thermo Fisher Scientific, USA) and maintained in a portable refrigerator freezer (−20°C) during transportation to the Indonesian Research Center for Veterinary Science, Bogor, Indonesia. Bats were released after sample collection.
Identification of bat species

Identification of bat species was performed using photo-documentation and external morphological (morphometric) measurements based on various indicators, such as forearm length, tibia length, hind leg length, ear length and shape, body length, body color, presence of claw on the second finger of the wing, tail length, body weight, shape of the muzzle and tongue, and color on the edge of the ear [7].

Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies directed against the nucleocapsid protein of SARS-CoV-2

ELISA was carried out using ID Screen SARS-CoV-2 Double Antigen Multi-species kits. Briefly, 25 μL of dilution buffer 13 was added to each well, 25 μL of the negative control solution was added to wells A1 and B1, 25 μL of the positive control solution was added to wells C1 and D1, and 25 μL of each sample to be tested were added to the remaining wells. The plates were covered and incubated for 45 min ± 5 min at 37°C (± 2°C). The wells were then emptied and washed 3 times with at least 300 μL of the kit's wash solution without drying the wells between washes. Conjugate 1X was prepared by diluting the concentrated conjugate 10-fold to 1:10 in dilution buffer 13, and 100 μL of conjugate 1X was added to each well. The plate was again covered and incubated for 30 min ± 3 min at 21°C (± 5°C). The wells were then emptied and washed 3 times with at least 300 μL of wash solution, avoiding drying of the wells between washes. Substrate solution (100 μL) was added to each well, and the plate was then covered and incubated for 20 min ± 2 min at 21°C (± 5°C) in the dark. Stop solution (100 μL) was added to each well, in the same order as described above to stop the reaction. Optical density was then read and recorded at 450 nm and the S/P percentage (S/P%) was calculated as:

\[ S/P\% = \frac{OD_{\text{sample}} - OD_{\text{NC}}}{OD_{\text{PC}} - OD_{\text{NC}}} \times 100 \]

Where OD_{PC} is optical density of positive control, OD_{NC} is optical density of negative control.

Samples presenting a S/P% less than or equal to 50% were considered negative. Those between 50% and 60% considered as doubtful, and those greater than or equal to 60% considered positive (interpretation: ≤ 50%, negative; 50%–60%, doubtful; ≥ 60%, positive).

Real-time reverse transcription polymerase chain reaction (rRT-PCR) targeting the nucleocapsid protein (N) gene of SARS-CoV-2

A 25-μL reaction mixture was established containing 5 μL of RNA and 12.5 μL of 2 × reaction buffer as provided with the Superscript III one-step RT-PCR system with Platinum Taq Polymerase (Invitrogen, USA) containing 0.4 mM of each deoxyribonucleotide triphosphates and 3.2 mM magnesium sulfate, 1 μL of reverse transcriptase/Taq mixture from the kit, and 1 μL for each primer probe (N1, N2, and RP). Thermal cycling was performed at 50°C for 15 min for reverse transcription, followed by 95°C for 2 min and 45 cycles of 95°C for 15 sec, 55°C for 30 sec using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA).

Reverse transcription polymerase chain reaction (RT-PCR) and partial DNA sequencing for BatCoV identification

RNA isolation and RT-PCR

The isolation of BatCoV genetic material obtained from individual field samples was carried out using The QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the kit instructions. The extracted RNA was stored at −20°C for RT-PCR. Genetic material identification was performed using primers designated for partial RdRp genes as described by Woo et al. [13].
The partial RdRp gene was amplified by performing RT-PCR using the AB9700 thermal cycler. The PCR mixture (25 µL) contained 12.5 µL of 2X reaction mix, 1 µL of each forward and reverse primer (20 pmol/µL), 0.5 µL SuperScript III RT/Platinum Taq Mix, and 10 µL of RNA template. The initial stage of RT-PCR was reverse transcription at 42°C for 45 min, followed by enzyme inactivation at 95°C for 3 min. Amplification included 35 cycles of denaturation for 30 sec at 95°C, annealing for 40 sec at 48°C, and elongation for 40 sec at 72°C. Final elongation was carried out at 72°C for 10 min. Amplicon products were visualized using 2% gel agarose, with gel electrophoresis run for 30 min at 100 V.

Partial DNA sequencing
PCR products were separated in 1% agarose by electrophoresis and the amplicon was excised and purified using a QIAquick gel purification kit (QIAGEN, Hilden, Germany). The sequencing method used was direct sequencing using a cycle sequencing kit (BigDye Terminator version 3.1; Applied Biosystems) on a Genetic Analyzer 3130 (Applied Biosystems). The nucleotide sequencing data obtained in this study were analyzed together with the genetic data available in the National Center for Biotechnology Information for the coronavirus RdRp gene. The production of multiple alignments of the gene and the analysis of the amino acids were carried out using BioEdit (http://www.mbio.ncsu.edu/BioEdit). Phylogenetic trees were generated by maximum likelihood (1,000 replicates) using the Tamura-Nei algorithm in MEGA version 5.2 (http://www.megasoftware.net). All isolates in this study were submitted to GenBank (www.ncbi.nlm.nih.gov) and given accession numbers MW652309–MW652323 and MZ451148–MZ451156.

RESULTS
A total of 182 rectal swab samples from 4 species of bats were acquired from bat collectors who were going to sell the bats to restaurants for consumption and from animal markets in several regencies/cities in Central Java Province (Surakarta City, Magelang Regency), Yogyakarta Province, and West Java Province (Bogor City, Cianjur Regency) in 2020 and 2021. Seventy-two BatCoV-positive bats (39.56%), based on PCR targeting the RdRp region, were detected among the 182 samples from 3 bat species: C. brachyotis, M. minimus, and R. amplexicaudatus (Table 1). Most of BatCoV-positive samples were detected from C. brachyotis and R. amplexicaudatus, which were the predominant bat species sampled in this study. BatCoV was also detected in 2 of the 5 M. minimus sampled. Of the 72 BatCoV-positive samples, the results for 24 were used in the phylogenetic analysis, namely IAARD-IRCVS DEP Bat 24, 26, 32, 39, 41; YOG Bat 06, 08, 09, 10, 14, 15, 17; WRT Bat 06, 07, 14 from 2020 and IAARD-IRCVS SJD K-05, 16, 21, 30, 35, 40, 45, 52, 55 from 2021 (Table 2).
Table 2. Bat coronavirus isolates sampled in Indonesia

| Isolate/sample name | Province/city/regency | Bat species/sources | Sampling years | Coronavirus species | NCBI accession number |
|---------------------|-----------------------|---------------------|----------------|-------------------|----------------------|
| IAARD-IRCVS DEP Bat 24 | Central Java/Surakarta | C. brachyotis/bat traders in live animal market | 2020 | Unclassified | MW652309 |
| IAARD-IRCVS DEP Bat 26 | Central Java/Surakarta | C. brachyotis/bat traders in live animal market | 2020 | Unclassified | MW652310 |
| IAARD-IRCVS DEP Bat 32 | Central Java/Surakarta | C. brachyotis/bat traders in live animal market | 2020 | Unclassified | MW652311 |
| IAARD-IRCVS DEP Bat 39 | Central Java/Surakarta | C. brachyotis/bat traders in live animal market | 2020 | Unclassified | MW652312 |
| IAARD-IRCVS DEP Bat 41 | Central Java/Surakarta | C. brachyotis/bat traders in live animal market | 2020 | Unclassified | MW652313 |
| IAARD-IRCVS YOG Bat 06 | Yogyakarta | C. brachyotis/bat traders/collectors | 2020 | Unclassified | MW652314 |
| IAARD-IRCVS YOG Bat 08 | Yogyakarta | C. brachyotis/bat traders/collectors | 2020 | Unclassified | MW652315 |
| IAARD-IRCVS YOG Bat 09 | Yogyakarta | C. brachyotis/bat traders/collectors | 2020 | Unclassified | MW652316 |
| IAARD-IRCVS YOG Bat 10 | Yogyakarta | C. brachyotis/bat traders/collectors | 2020 | Unclassified | MW652317 |
| IAARD-IRCVS YOG Bat 14 | Yogyakarta | C. brachyotis/bat traders/collectors | 2020 | Unclassified | MW652318 |
| IAARD-IRCVS YOG Bat 15 | Yogyakarta | C. brachyotis/bat traders/collectors | 2020 | Unclassified | MW652319 |
| IAARD-IRCVS YOG Bat 17 | Yogyakarta | C. brachyotis/bat traders/collectors | 2020 | Unclassified | MW652320 |
| IAARD-IRCVS WRT Bat 06 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2020 | Unclassified | MW652321 |
| IAARD-IRCVS WRT Bat 07 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2020 | Unclassified | MW652322 |
| IAARD-IRCVS WRT Bat 14 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2020 | Unclassified | MW652323 |
| IAARD-IRCVS SJD K-05 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45148 |
| IAARD-IRCVS SJD K-16 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45149 |
| IAARD-IRCVS SJD K-21 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45150 |
| IAARD-IRCVS SJD K-30 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45151 |
| IAARD-IRCVS SJD K-35 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45152 |
| IAARD-IRCVS SJD K-40 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45153 |
| IAARD-IRCVS SJD K-45 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45154 |
| IAARD-IRCVS SJD K-52 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45155 |
| IAARD-IRCVS SJD K-55 | West Java/Cianjur | R. amplexicaudatus/bat collectors | 2021 | Unclassified | MZ45156 |

NCBI, National Center for Biotechnology Information.

Forty-four rectal swab samples were obtained from bats at animal markets in Surakarta City. Twelve BatCoV-positive samples were detected among the 21 C. brachyotis sampled. Bat samples collected from the traders/collectors in Yogyakarta Province showed that 1 of 1 R. amplexicaudatus, 12 of 24 C. brachyotis, and 2 of 5 M. minimus carried BatCoV. In addition, 45 of 95 R. amplexicaudatus obtained from collectors in Cianjur Regency were BatCoV-positive. In contrast, BatCoV was not detected in all samples from 23 P. vampyrus collected from animal markets in Surakarta City, 4 from animal markets in Magelang Regency, 5 from Yogyakarta Province, and 4 from bat traders in Bogor City.

Phylogenetic analysis (Fig. 1) showed that 24 of the BatCoV-positive samples belonged to the subfamily Coronavirinae within the family Coronaviridae that were clustered with other unclassified Bat betaCoV namely Kenya bat coronavirus/BtKY56/BtKY55, Betacoronavirus E.isa/M/Spain/2007, Coronavirus PREDICT CoV-24, Bat coronavirus BtCoV/UKR-G17/ Pip_nat/UKR/2011, and Betacoronavirus H.sav/Italy/Spain/2007. Some of the positive samples in this study were obtained from traders at animal markets and bat collectors that supply bat meat for restaurants. Interactions that occur between bats, other animals, and humans may potentially become the source of zoonotic disease transmission, as illustrated in Fig. 2.

Related to close contact with humans, serological and rRT-PCR investigations were also conducted to investigate the possible circulation of SARS-CoV-2-related coronaviruses in bats in Indonesia. The samples tested produced negative results in both the ELISA tests to detect antibodies against the N protein of the SARS-CoV-2 and the rRT-PCR assays targeting the nucleocapsid protein (N) gene of SARS-CoV-2 (Tables 3 and 4).
DISCUSSION

Bats are widely distributed globally, although each species has a specific geographic area [26]. Woo et al. [27] reported that only alpha- and betaCoV have been identified in bats. Bat betaCoV was previously reported on D. moluccensis collected from the Paguyaman Regency, Gorontalo Province, Indonesia. However, D. moluccensis is a native bat species from Moluccas, Indonesia [6]. The phylogenetic tree analysis indicated that the BatCoVs in the previous study formed distinct branches but were closely related to BatCoV HKU9, HKU9-2, HKU9-5-2, and HKU9-10-2 originating from China and BatCoV KY06 from Kenya [24]. In 2018, BatCoVs were detected in P. alecto from the Gorontalo Province, Indonesia. Those 3 BatCoVs
isolates (INDSBT101, INDSBT102, INDSBT103) have 98% nucleotide similarity with Indonesian isolates, namely Bat Coronavirus Indonesia IFB 2012-8F D. moluccensis [24], meanwhile the 5 other isolates (INDSBT110, INDSBT192, INDSBT198, INDSBT180, INDSBT195) have 85%–88% nucleotide similarity with Bat Coronavirus BtCoV/B55762/S. hea/CB/Tha/6/2012 isolated from Scotophilus heathii in Thailand [25].

Indonesia is reported to have 81 species of bats that belong to suborder Megachiroptera (fruit nectar-eating bats) and 158 species in the suborder Microchiroptera (non-fruit-nectar-eating bats). As many as 10 families of bats have been reported in Indonesia, including Pteropodidae, which belongs to the Megachiroptera suborder, and Rhinopomatidae, Emballonuridae, Nycteridae, Megadermatidae, Rhinolophidae, Hipposideridae, Vespertilionidae, Miniopteraidae, and Molossidae families that belong to the Microchiroptera suborder. As an archipelago country, Indonesia has the highest number of fruit- and nectar-eating bat species in the world. Fruit-nectar bats are very important for the process of pollination, fertilization, and seed dispersal. There are at least 21 genera and 81 species that belong to Pteropodidae family [6,7,28], and, in this study, 3 species were observed to carry BatCoV (C. brachyotis, R. amplexicaudatus, and M. minimus).

| Positive/negative result | No. of bat sera samples tested for the nucleocapsid protein of the SARS-CoV-2 |
|-------------------------|---------------------------------------------------------------------------------|
| Positive                | 0                                                                               |
| Negative                | 38                                                                              |
| Total                   | 38                                                                              |

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
Table 4. Real-time polymerase chain reaction results for samples tested for presence of the nucleocapsid protein (N) gene of severe acute respiratory syndrome coronavirus 2 virus

| Sample name                  | Result         | Cycle threshold value (N1 gene/N2 gene) |
|------------------------------|----------------|----------------------------------------|
| IAARD-IRCVS DEP Bat 24       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS DEP Bat 26       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS DEP Bat 32       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS DEP Bat 39       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS DEP Bat 41       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS YOG Bat 06       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS YOG Bat 08       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS YOG Bat 09       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS YOG Bat 10       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS YOG Bat 14       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS YOG Bat 17       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS WRT Bat 06       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS WRT Bat 07       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS WRT Bat 14       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS WRT Bat 25       | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-01         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-02         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-03         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-04         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-05         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-06         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-07         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-08         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-09         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-10         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-11         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-12         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-13         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-14         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-15         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-16         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-17         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-18         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-19         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-20         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-21         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-22         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-23         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-24         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-25         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-26         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-27         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-28         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-29         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-30         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-31         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-32         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-33         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-34         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-35         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-36         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-37         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-38         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-39         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-40         | Negative       | Undetermined/Undetermined               |
| IAARD-IRCVS SJD K-41         | Negative       | Undetermined/Undetermined               |

(continued to the next page)
The discovery of BatCoV in several species of bats in Indonesia suggests that bats are potential natural reservoir hosts for coronavirus and may transmit that virus to other species, such as occurred in previous infectious disease outbreaks such as SARS-CoV and MERS-CoV [23,29]. Bats are reported to possess efficient and varied antiviral responses associated with adaptations in their immune system and their ability to evolve. The adaptive immune mechanism in bats can suppress the pathological effects of the inflammation caused by viral infection. However, various factors, such as stress, may contribute to unbalancing the mechanism, resulting in increased viral replication and shedding, and potentially becoming a source of cross-species virus transmission, including human transmission [11].

Live animal markets are considered a major area for the spread of zoonotic diseases. Several zoonotic diseases have originated from live animal markets, including SARS-CoV and avian influenza virus [30]. Transportation, cages, and environmental conditions at live animal markets may trigger stress responses in various animals, including bats. Under stress conditions, animals tend to be more vulnerable to viruses and infections. In addition, cage systems, which are often stacked, may facilitate efficient transmission of the virus among animals [31].

In Indonesia, bats and other animals are widely traded in several live animal markets. During studies that we conducted in several animal markets in Indonesia, we observed that bats are kept close to other animals, including various species of poultry, other birds, as well as various mammals and reptiles. Collectors capture bats in forests or caves and sell them to traders in the animal markets and to restaurant owners. Bats may be held together with other animals for several days to months prior to being sold (Fig. 3). Bat meat is commonly consumed among local communities due to traditional beliefs that such meat can increase stamina and cure particular diseases, such as asthma [22]. Bushmeat trade-related activities are considered to be connected to the transmission of zoonotic diseases. In addition to the people who consume bushmeat, people who slaughter, cut the body or organs, or have direct contact with the blood, urine, and feces, and those who may be scratched or bitten by wild animals have a high potential of being infected by zoonotic disease agents. Of all emerging diseases, zoonoses have been identified as the most significant increasing threat to global health [14].

---

**Table 4. (Continued) Real-time polymerase chain reaction results for samples tested for presence of the nucleocapsid protein (N) gene of severe acute respiratory syndrome coronavirus 2 virus**

| Sample name       | Result       | Cycle threshold value (N1 gene/N2 gene) |
|-------------------|--------------|----------------------------------------|
| IAARD-IRCVS SJD K-42 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-43 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-44 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-45 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-46 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-47 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-48 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-49 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-50 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-51 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-52 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-53 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-54 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-55 | Negative     | Undetermined/Undetermined              |
| IAARD-IRCVS SJD K-56 | Negative     | Undetermined/Undetermined              |
| Positive control  | Positive     | 34.08/33.13                            |
| No template control | Negative     | Undetermined/Undetermined              |

The detection of bat coronaviruses in Indonesia suggests that bats are potential natural reservoir hosts for coronavirus and may transmit that virus to other species, such as occurred in previous infectious disease outbreaks such as SARS-CoV and MERS-CoV [23,29]. Bats are reported to possess efficient and varied antiviral responses associated with adaptations in their immune system and their ability to evolve. The adaptive immune mechanism in bats can suppress the pathological effects of the inflammation caused by viral infection. However, various factors, such as stress, may contribute to unbalancing the mechanism, resulting in increased viral replication and shedding, and potentially becoming a source of cross-species virus transmission, including human transmission."
This study shows that 3 species of bats, *C. brachyotis*, *M. minimus*, and *R. amplexicaudatus*, that were collected by bat traders/bat collectors and examined in this study were found to carry viruses of an unclassified Bat coronavirus phylogenetic group. The ELISA results did not reveal antibodies against the nucleocapsid protein of the SARS-CoV-2 and the rRT-PCR assay results did not show the presence of SARS-CoV-2 viral RNA. Close contacts between humans, bats, and other animals have a high potential for transmitting zoonotic diseases. Further studies regarding coronaviruses carried by bats or other animals and the possible effects of environmental conditions are needed to identify possible novel virus transmission routes, particularly in live animal markets. Early detection of pathogen transmission and the application of appropriate control measures may minimize the destructive impacts on global health. Thus, more surveillance studies are needed to investigate the potentially important role of bats as natural reservoir hosts in the interspecies transmission of coronaviruses.

**ACKNOWLEDGMENTS**

The authors express their appreciation and thanks to Teguh S, Ace ES, Suraida Meisari DVM, Zaki Aminullah DVM for technical assistance and the agricultural services of the respective regencies/cities staff for field activity assistance. Ni Luh Putu Indi Dharmayanti and Diana Nurjanah are the main authors and contributed equally to this article.

**REFERENCES**

1. Voigt CC, Kingston T. Bats in the anthropocene. In: *Bats in the Anthropocene: Conservation of Bats in a Changing World*. Cham: Springer International Publishing; 2016, 1-9.
2. O’Shea TJ, Cryan PM, Cunningham AA, Fooks AR., Hayman DT, Luis AD, et al. Bat flight and zoonotic viruses. Emerg Infect Dis. 2014;20(5):741-745.

3. Sendow I, Field H, Adjid RMA, Syafrizati T, Darminto D, Morrissy C, et al. Seroepidemiologi Nipah virus pada kalong dan ternak babi di beberapa wilayah di Indonesia, J Biologi Indone. 2008;5(1):35-44.

4. Sendow I, Field H, Ratnawati A, Adjid RMA, Saepulloh M, Breed A, et al. Status infeksi virus hendra pada kalong (Pteropus Spp.) Di Pontianak, Kalimantan Barat Dan Manado, Sulawesi Utara. J Biologi Indone. 2013;9(1):31-38.

5. Anthony SJ, Johnson CK, Greig DJ, Kramer S, Che X, Wells H, et al. Global patterns in coronavirus diversity. Virus Evol. 2017;3(1):vex012.

6. Anthoney SJ, Johnson CK, Greig DJ, Kramer S, Che X, Wells H, et al. Global patterns in coronavirus diversity. Virus Evol. 2017;3(1):vex012.

7. Maryanto I, Achmadi AS, Sulistyadi E, Wiantoro S, Maharadatunkamsi, Yoneda M, et al. Checklist of The Mammals of Indonesia: Scientific, English, Indonesia Name and Distribution Area Table in Indonesia Including CITES, IUCN and Indonesian Category for Conservation, Vol. 3. Bogor: Puslitbang Biologi LIPI; 2019.

8. Wong AC, Li X, Lau SK, Woo PC. Global epidemiology of bat coronaviruses. Viruses. 2019;11(2):174.

9. Dinde AO, Mobio AJ, Konan AG, Fokou G, Yao K, Esso EL, et al. Response to the Ebola-related bushmeat consumption ban in rural Côte d’Ivoire. Agirc Food Secur. 2017;6(1):28.

10. Vats R, Thomas S. A study on use of animals as traditional medicine by Sukuma Tribe of Busega District in North-western Tanzania. J Ethnobiol Ethnomed. 2015;11(1):38.

11. Subudhi S, Rapin N, Misra V. Immune system modulation and viral persistence in bats: understanding viral spillover. Viruses. 2019;11(2):192.

12. Monagin C, Paccha B, Liang N, Trufan S, Zhou H, Wu D, et al. Serologic and behavioral risk survey of workers with wildlife contact in China. PLoS One. 2018;13(4):e0194647.

13. Woo PC, Lau SK, Li KS, Poon RW , Wong BH, Tsoi HW , et al. Molecular diversity of coronaviruses in bats. Virology. 2006;351(1):180-187.

14. Kurpiers LA, Schulte-Herbrüggen B, Ejotre I, Reeder DM. Bushmeat and emerging infectious diseases: lessons from Africa. In: Angelici FM, editor. Problematic Wildlife: a Cross-Disciplinary Approach. Cham: Springer International Publishing; 2016, 507-551.

15. Memish ZA, Cotten M, Meyer B, Watson SJ, Alsahafi AJ, Al Rabeeah AA, et al. Human infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013. Emerg Infect Dis. 2014;20(6):1012-1015.

16. Han HJ, Yu H, Yu XI. Evidence for zoonotic origins of Middle East respiratory syndrome coronavirus. J Gen Virol. 2016;97(2):274-280.

17. Wang LF, Eaton BT. Bats, civets and the emergence of SARS. Curr Top Microbiol Immunol. 2007;315:325-344.

18. Chan JF, Kok KH, Zhu Z, Chu H, To KK, Yuan S, et al. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. Emerg Microbes Infect. 2020;9(1):221-226.

19. Zhou P, Yang XL, Wang KG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a novel coronavirus of probable bat origin. Nature. 2020;579(7798):270-273.

20. Ding Y, He L, Zhang Q, Huang Z, Che X, Hou J, et al. Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. J Pathol. 2004;203(2):622-630.

21. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. Lancet. 2020;395(10223):514-523.
Detection of bat coronaviruses in Indonesia

22. Sheherazade, Tsang SM. Quantifying the bat bushmeat trade in North Sulawesi, Indonesia, with suggestions for conservation action. Glob Ecol Conserv. 2015;3:324-330.

23. Wang LF, Shi Z, Zhang S, Field H, Daszak P, Eaton BT. Review of bats and SARS. Emerg Infect Dis. 2006;12(12):1834-1840.

24. Anindita PD, Sasaki M, Seriyono A, Handharyani E, Orba Y, Kobayashi S, et al. Detection of coronavirus genomes in Moluccan naked-backed fruit bats in Indonesia. Arch Virol. 2015;160(4):1113-1118.

25. Febriani W, Saepuloh U, Ayuningsih E, Saputra R, Purbatapsila A, Nangoy M, et al. Bat coronavirus of *Pteropus alecto* from Gorontalo Province, Indonesia. Int J Trop Vet Biomed Res. 2018;3(2):36-42.

26. Morgan CN, Ammerman LK, Demere KD, Doty JB, Nakazawa YJ, Mauldin MR. Field identification key and guide for bats of the United States of America. Occas Pap Tex Tech Univ Mus. 2019;360:360.

27. Woo PC, Lau SK, Lam CS, Lau CC, Tsang AK, Lau JH, et al. Discovery of seven novel mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J Virol. 2012;86(7):3995-4008.

28. Yuladi B, Sari TF, Handayani FD. *Kelelawar Sulawesi Jenis dan Peranannya*. Jakarta: Penerbit Badan Penelitian dan Pengembangan Kesehatan; 2018.

29. van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. mBio. 2012;3(6):e00473-12.

30. Chomel BB, Belotto A, Meslin FX. Wildlife, exotic pets, and emerging zoonoses. Emerg Infect Dis. 2007;13(1):611.

31. Aguirre AA, Catherina R, Frye H, Shelley L. Illicit wildlife trade, wet markets, and COVID-19: preventing future pandemics. World Med Health Policy. 2020;12(3):256-265.