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Molecular detection of bat coronaviruses in three bat species in Indonesia

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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.

Keywords: Coronavirus; zoonoses; bats; Indonesia

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

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

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.

of viruses into the environment [11]. 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 amplexicaudatus* (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:

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

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].

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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 version 7 (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 1. Detection of bat coronavirus-positive samples for reverse transcription polymerase chain reaction of the partial RdRp region in several bat species in Indonesia

Species	Surakarta City		Magelang Regency		Yogyakarta Province		Bogor City		Cianjur Regency	
	No	Positive	No	Positive	No	Positive	No	Positive	No	Positive
R. amplexicaudatus	-	-	-	-	1	1	-	-	95	45
P. vampyrus	23	0	4	0	5	0	4	0	-	-
C. brachyotis	21	12	-	-	24	12	-	-	-	-
M. minimus	-	-	-	-	5	2	-	-	-	-

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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	MZ451148
IAARD-IRCVS SJD K-16	West Java/Cianjur	*R. amplexicaudatus*/bat collectors	2021	Unclassified	MZ451149
IAARD-IRCVS SJD K-21	West Java/Cianjur	*R. amplexicaudatus*/bat collectors	2021	Unclassified	MZ451150
IAARD-IRCVS SJD K-30	West Java/Cianjur	*R. amplexicaudatus*/bat collectors	2021	Unclassified	MZ451151
IAARD-IRCVS SJD K-35	West Java/Cianjur	*R. amplexicaudatus*/bat collectors	2021	Unclassified	MZ451152
IAARD-IRCVS SJD K-40	West Java/Cianjur	*R. amplexicaudatus*/bat collectors	2021	Unclassified	MZ451153
IAARD-IRCVS SJD K-45	West Java/Cianjur	*R. amplexicaudatus*/bat collectors	2021	Unclassified	MZ451154
IAARD-IRCVS SJD K-52	West Java/Cianjur	*R. amplexicaudatus*/bat collectors	2021	Unclassified	MZ451155
IAARD-IRCVS SJD K-55	West Java/Cianjur	*R. amplexicaudatus*/bat collectors	2021	Unclassified	MZ451156

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/J/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**).

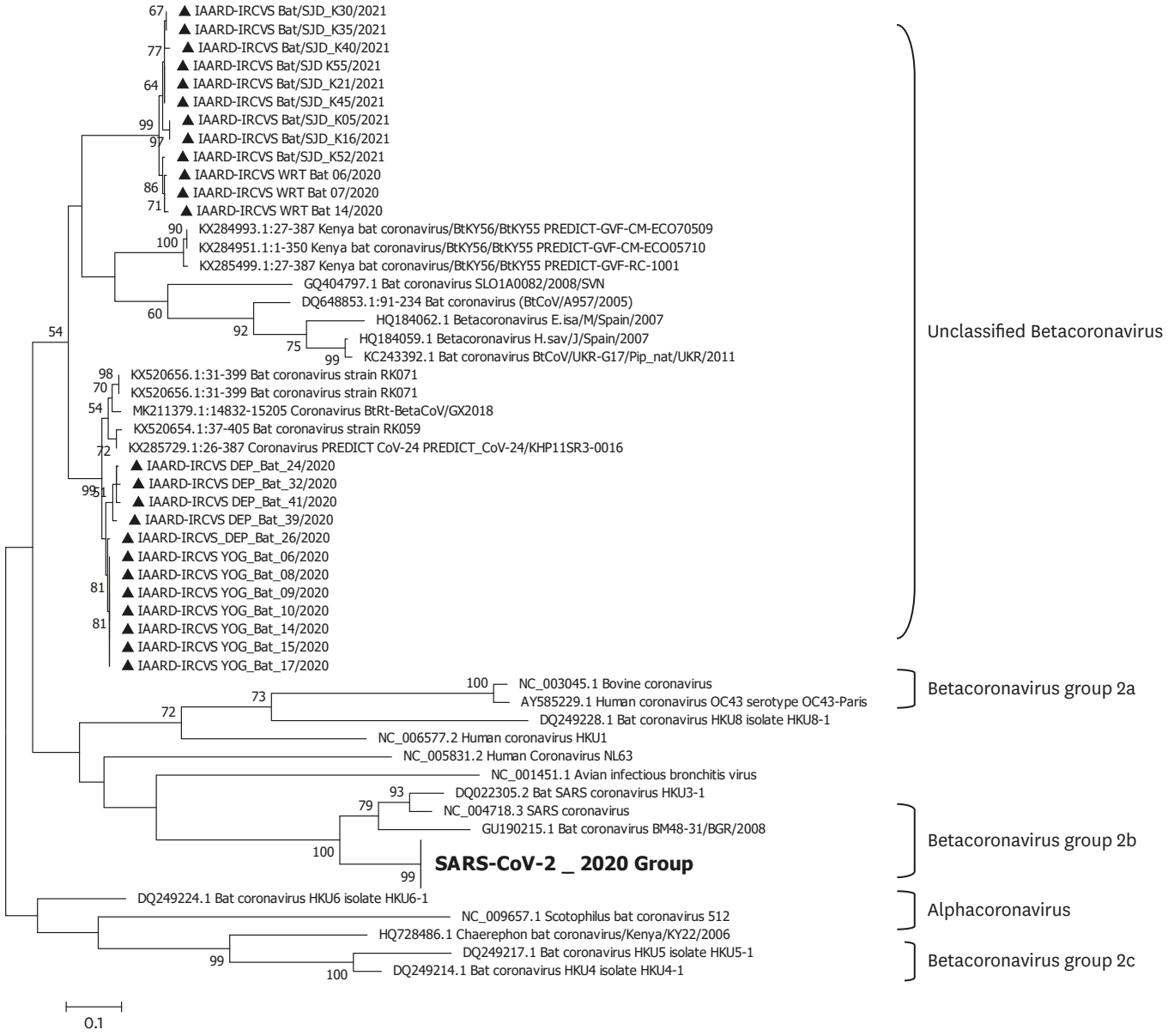

Fig. 1. Phylogenetic tree of the partial RdRp gene of bat coronavirus. The isolates used in this study are indicated by a black triangle shape.
SARS-CoV-2, severe acute respiratory syndrom coronavirus 2.

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

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Fig. 2. Interactions that may occur between humans and bats that may be caged together with other animals in a live animal market.

isolates (INDSWBT101, INDSWBT102, INDSWBT103) have 98% nucleotide similarity with Indonesian isolates, namely Bat Coronavirus Indonesia IFB 2012-8F *D. moluccensis* [24], meanwhile the 5 other isolates (INDSWBT110, INDSWBT192, INDSWBT198, INDSWBT180, INDSWBT195) 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*, *Hiposideridae*, *Vespertilionidae*, *Miniopterеridae*, 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*).

Table 3. Enzyme-linked immunosorbent assay results for bat sera samples examined to detect antibodies against the nucleocapsid protein of the SARS-CoV-2

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.

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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)
	Positive	Negative	
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 15		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)

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)
	Positive	Negative	
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 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].

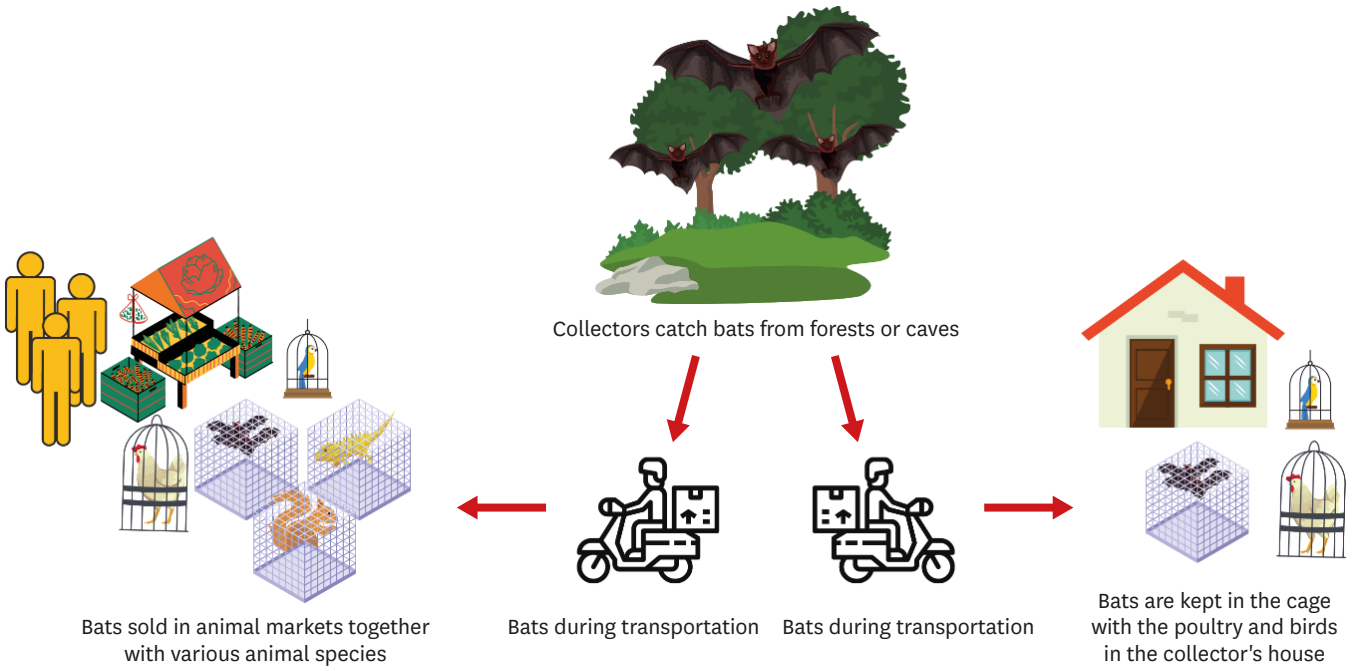

Fig. 3. Description of how bats in this study were captured, transported, and sold by bat collectors. Collectors captured various species of bats from forests and caves, transported the bats to a markets or their home by motorcycle. People/buyers purchased bats from traders at live animal markets or directly from the collector's house.

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.

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