



RESEARCH ARTICLE

Presence of SARS-CoV-2-like coronaviruses in bats from east coast Malaysia

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ABSTRACT

Most of the public health importance coronaviruses, such as Severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2 are likely originated from bats and spread to humans through intermediate hosts; civet cats, dromedary camel and Malayan pangolin, respectively. SARS-CoV-2-like coronaviruses were detected in Thailand, which is neighbouring with Kelantan in East Coast Malaysia. To date, there is no report on the presence of public health concerns (SARS-CoV, SARS-CoV-2 and MERS-CoV) coronaviruses in bats from Malaysia. This study was aimed to elucidate the presence of these coronaviruses in bat samples from East Coast, Malaysia. A total of hundred seventy oropharyngeal swab samples were collected from three states of East Coast Malaysia. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was conducted based on partial 3' Untranslated region (3'UTR) or ORF10 gene and the products were sequenced. The sequences were compared with all coronavirus sequences from the National Center for Biotechnology Information-GenBank (NCBI-GenBank) using NCBI-Basic Local Alignment Search Tool (NCBI-BLAST) software. A phylogenetic tree was constructed to determine the genetic relationship among the detected coronaviruses with the reference coronaviruses from the NCBI-GenBank. Our results showed that SARS-CoV-2-like viruses were present in 3% (5/170) of the bats from East Coast Malaysia that have 98-99% sequence identities and are genetically related to SARS-CoV-2 from humans. This finding indicates the presence of SARS-CoV-2-like viruses in bats from East Coast Malaysia that may become a public health concern in the future.

Keywords: SARS- CoV-2 like coronaviruses; bats; East Coast Malaysia.

INTRODUCTION

Coronaviruses (CoVs) are a single-stranded ribonucleic acid (RNA) viruses which classified into four genera; *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*, and both *Alphacoronavirus* and *Betacoronavirus* houses CoVs of public health importance (Aravena *et al.*, 2022). It is shown that bats are the major hosts for alphacoronaviruses and betacoronaviruses (Hu *et al.*, 2015). Sarbecoviruses and Merbecovirus in the *betacoronavirus* genus have been associated with three public health concerns coronavirus to humans in the past 2 decades (Tan *et al.*, 2021). First outbreak was caused by severe acute respiratory syndrome coronavirus (SARS-CoV), where it occurred in 2003 and spread across Guangdong, China, infecting more than 8,000 people and killing 774 throughout the globe (Christopher *et al.*, 2004). Ten years later, the Middle East respiratory syndrome CoV (MERS-CoV) virus, first appeared in Saudi Arabia in 2012 which was responsible for almost 2000 cases and 803 fatalities in 27 nations (Ramadan & Shaib, 2019). The latest one is SARS-CoV-2, the causative agent of the previous pandemic and current pandemic COVID-19, which has claimed more than 6.6 million lives and infected more than 654.6 million people as of December 13, 2022 (WHO, 2022). SARS-CoV

and SARS-CoV-2 are classified into Sarbecoviruses and MERS-CoV in Merbecovirus subgenus (Aravena *et al.*, 2022). Bat coronaviruses have been identified as the origin of these public health concern coronaviruses due to their almost similar genetic makeup (Hu *et al.*, 2015) with the involvement of palm civets, camels and Malayan pangolin as an intermediate host for SARS-CoV, MERS-CoV, and SARS-CoV-2 coronaviruses, respectively (Decaro & Lorusso, 2020; Stout *et al.*, 2020). According to recent estimates, bats are estimated to have up to 3204 coronaviruses, many of which have yet to be identified and may have a zoonotic potential in the future (Zappulli *et al.*, 2020). SARS-CoV-2-like coronaviruses have been reported in bats from Laos (Mallapaty, 2021) Cambodia (Delaune *et al.*, 2021) and Thailand (Wacharapluesadee *et al.*, 2021) that shares a border with one of the states in the East Coast, Kelantan, Malaysia. Tan *et al.* (2021) have identified 10 CoV RNAs from 21 bat samples from Sarawak, Malaysia which were closely related to Decacovirus-1 and Decacovirus-2, Sarbecovirus, and an unclassified CoV, but according to them, all of their bat coronaviruses were distant from the currently known public health concerns coronaviruses. Therefore, it is significant to determine the presence of these public health coronaviruses in bats from Malaysia especially Kelantan for the prediction and prevention of another pandemic emergence in the future.

MATERIALS AND METHOD

Ethics statement and permit approval

All sampling procedures were performed with an approval from the Animal Ethics Committee of the University Malaysia Kelantan (UMK) with the ethic number UMK/FPV/ACUC/PG/6/2021. The study was conducted in accordance with the Guide for the Care and Use of Wild Mammals from the Department of Wildlife and National Parks Malaysia with an approval permit obtained from this department.

Sampling sites

Due to the Covid-19 situation, only 170 oropharyngeal swabs from various bat species (49 *Hipposideros* spp, 57 *Rhinolopus* spp, 43 *Eonycteris* spp, 13 *Cynopterus* spp, 3 *Balionycteris* spp, 2 *Myotis* spp, 2 *Nycteris* spp, 1 *Kerivoula* spp) (Table 1) were collected from August to October 2021. They were collected from three states in East Coast Malaysia; Kelantan (5.7158° N, 101.7444° E, Gunung Reng), Terengganu (4.9672° N, 102.9579° E, Hutan Lipur Sekayu), and Pahang (4.6928916° N, 102.00433 101.7444° E, Taman Negara, Merapoh) (Figure 1). Samplings were done mostly at the entrance

of the cave, area that has a body of water, and vicinity of the cave that has fruits. Sampling location in Kelantan (Gunung Reng) was chosen due to its close proximity to the Thai border, where SARS-CoV-2-related coronaviruses have been reported in bats and pangolins and possible migration of bats to this location is high. These sites were also selected based on advices from experienced bat zoologists from Universiti Malaysia Kelantan and University Malaysia Terengganu on the presence of big population of bats in these areas, which would increase the likelihood to obtain more samples during a limited period of time due to Covid-19 movement control order (MCO). The sampling is done around 7 pm until 3 am depending on the number of bats being captured. Sampling at night time was preferred to reduce stress to the bats due to heat and light during daytime. Method of capturing bats was done by placing a mist and a harp net at the entrance of the roosting site or at a suspected flight pathway of the bats. These nets were placed before sunset and removed before sunrise. Oropharyngeal swabs were taken while being physically restrained by trained personnel fully equipped with PPE and the bats were released back to the environment after the swabs were taken.

Table 1. Samples collected from bat species and States in East Coast, Malaysia

	Merapoh, Pahang	Gunung Reng, Kelantan	Hutan lipur Sekayu, Terengganu	Number of bats
<i>Hipposideros</i> spp.	29	6	14	49
<i>Rhinolopus</i> spp.	1	37	19	57
<i>Eonycteris</i> spp.		43		43
<i>Cynopterus</i> spp.			13	13
<i>Balionycteris</i> spp.			3	3
<i>Myotis</i> spp.			2	2
<i>Nycteris</i> spp.		2		2
<i>Kerivoula</i> spp.			1	1
Total	30	88	52	170

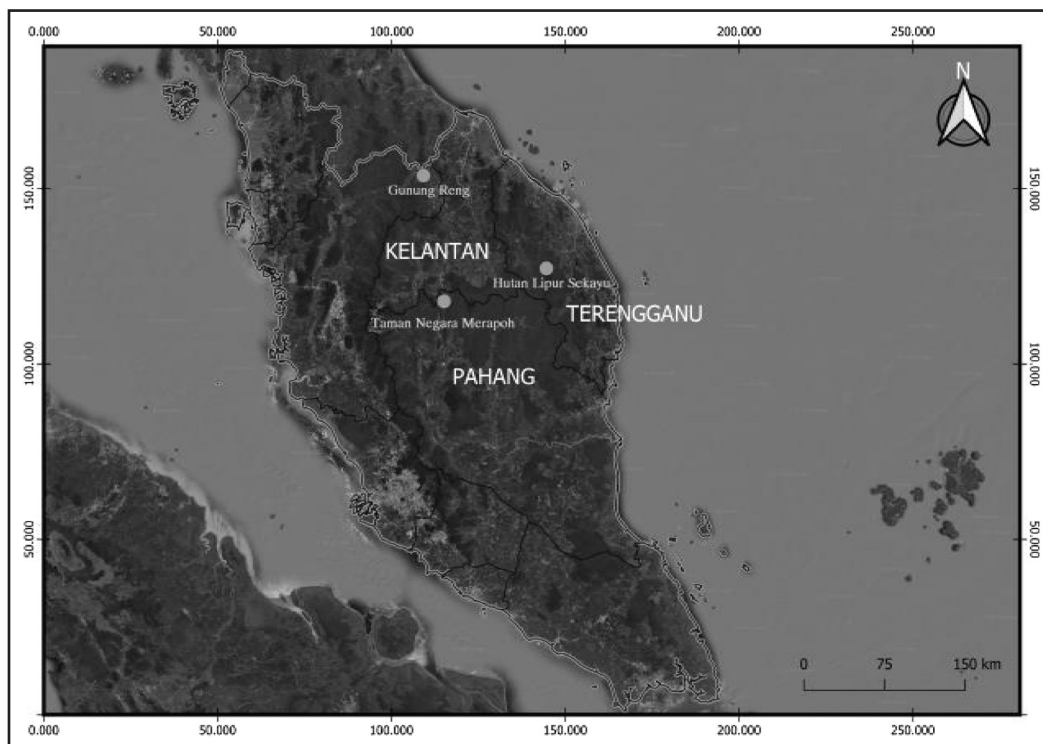


Figure 1. Map of Malaysia depicting the point of sampling sites in Kelantan (Gunung Reng), Terengganu (Hutan Lipur Sekayu), and Pahang (Taman Negara, Merapoh). Map was produced using QGIS Version 3.20.3 (<http://qgis.org/en/site/>).

The swab samples were placed in a viral transport medium (Dulbecco's Modified Eagle Medium with 1% penicillin-streptomycin, 10000U/mL), stored in -20°C and transported on ice to the virology laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (UMK). The personnel involved in sampling was equipped with necessary personal protective equipment (PPE) such as face masks, safety boots and coveralls to avoid possible transmission of the virus to the handlers.

Ribonucleic acid (RNA) extraction and Reverse transcription polymerase chain reaction (RT-PCR) for coronavirus detection

Viral RNA was extracted directly from 200µl of the swab samples in viral transport medium using GENEzol reagent following the manufacturer's protocol (Geneaid Biotech Ltd, Taiwan). The purity and concentration of the RNA were checked using a NanoPhotometer (Implen, USA). The RNA was eluted in 30µl of nuclease-free water and used as the template for RT-PCR. Control virus of human SARS-CoV-2 positive case was obtained from one of the hospitals in Kelantan, Malaysia. Conventional RT-PCR was performed using published universal and designed primers as listed in Table 2 for the detection of coronaviruses. The CoV F and CoV R primers were designed manually by aligning the reference sequences of 3'UTR (ORF10) of MERS-CoV, SARS-CoV, SARS-CoV-2 and bat coronaviruses from the National Center for Biotechnology Information GenBank (NCBI-GenBank) using ClustalW program (GenomeNet, Kyoto University Bioinformatic Center). The specificity of the primers was checked using NCBI-Basic Local Alignment Search Tool (NCBI-BLAST) against all the sequences from the NCBI-GenBank. The 3' UTR gene was used for the designed primers because this gene is conserved (Chan *et al.*, 2020) and also based on high detection rate of an avian coronavirus, infectious bronchitis virus (IBV) in Malaysia compared to other genes (Mohamed, 2000). RT-PCR reaction was conducted using thermocycler (Biorad T100, USA) in a total volume of 25µl reaction mixture containing 1X AccessQuick RT-PCR reagent kit (Promega, USA), five µl preheated RNA at 95°C for 5 min and 0.2 uM of each forward and reverse primers. The reaction was carried out at 42°C for 1 h, 95°C for 5 min, 35 cycles of 92°C for 1 min, 50°C for 1 min and 72°C for 1 min, and one cycle of 72°C for 5 min for

final elongation. The products were run at 100 Volt for 50 minutes in 2% Midori green (Nippon Genetics, Europe) stained agarose gel and visualised using Gel DocTM EZ Gel Imager (Bio-Rad, USA).

Sequence analysis

RT-PCR products of the samples with an expected band size were then sent to Apical Sdn. Bhd., Malaysia for the sequencing reaction. The sequences were edited using Bioedit 7.2 software (Informer Technologies, Inc) and compared with all the coronavirus sequences in the Genbank using NCBI-BLAST software and aligned using ClustalW program to determine the sequence identity and similarity.

Phylogenetic analysis of bat coronaviruses

Multiple alignment of the sequences was prepared using the ClustalX 2.1 program (Thompson *et al.*, 1997) and phylogenetic analysis was conducted by neighbour-joining methods using Neighbour Joining (NJ) software (Perrière & Goul, 1996). Sixteen reference 3'UTR sequences of coronaviruses from humans (Hu-Wu_CoV_2019-NC_045521, Hu_US_Ari_2021-OM134814, Hu_US_Colo_2022-OP982032, SARS-CoV_Tor2-NC004718, MERS-CoV_Riyadh-OL622035), bats (BatYunnan_RpYN06-MZ081381, BatThai_RacCS203-MW251308, BatLao_BANAL-20-236-MZ937003, BatKenya_BtKY72-KY352407, BatCoV_RaTG13-MN996532.2, BatChina_WIV-NC004718, batYunnan_RsYN03-MZ081379, BatMY_CoV51-MZ293757, BatCoV_HKU4-NC_009019), and pangolin (PangolinChina_MP789-MT121216) were retrieved from the NCBI-GenBank with bat Alphacoronavirus from Finland, Alpha_BtCoV/020_16-NC_076629 used as an outgroup to construct the tree. Some of the bat coronaviruses cannot be included in this study due to the unavailability of 3'UTR sequences in the NCBI GenBank.

RESULTS

RNA purity and concentration

Concentration and purity of extracted RNAs were within the concentration 0.1-0.2 mg and purity >2 at the 260/280 nm ratio which were within the range for use in RT-PCR reaction.

Table 2. List of primers used in RT-PCR reaction

Primer	Sequence (5'...3')	Gene	Expected Size (bp)	Reference
Pan_CoV_F1	GGTTGGGAYTAYCCHAARTGYGA	RNA-dependent	440 bp	Holbrook <i>et al.</i> , 2021
Pan_CoV_R1	CCRTCATCAGAHARWATCAT	RNA polymerase		
Pan_CoV_R2	CCRTCATCACTHARWATCAT	(RdRp) gene		
Pan_CoV_F2	GAYTAYCCHAARTGTGAYAGA		430 bp	
Pan_CoV_F3	GAYTAYCCHAARTGTGAYMGH			
CoV-FWD3	CGTTGGIACWAAYBTVCWYTCARBTRGG	RNA-dependent	440 bp	Quan <i>et al.</i> , 2010
CoV-RVS3	GGTCATKATAGCRTCAVMASWWGCNACNACATG	RNA polymerase		
CoV-FWD4	GGCWCCWCHGGNGARCAATT	(RdRp) gene		
CoV-RVS3	GGWAWCCCAAYTGYTGWAYRTC			
CoV F	TCTACTCTTRYRCAGAATG	3' Untranslated	120	Designed Primers
CoV R	GTGGYTCTTTMAMDMTCTC	region (3' UTR)		

Reverse transcription polymerase chain reaction (RT-PCR) for the presence of coronavirus

Out of 170 samples, five samples (3%) were positive for the presence of coronavirus using designed CoV F and CoV R primers that cover the partial 3'UTR gene of coronaviruses. No band was observed using the published primers based on RNA-dependent RNA polymerase (RdRp) gene. One positive sample (R63) was from *Eonycteris* spp. (Cave nectar bat) from Gunung Reng, Kelantan, two (S7 and S11) from *Rhinolopus* spp. (Horseshoe bat), one from each *Balionycteris* spp. (S13) and *Hipposideros larvatus* (Insectivorous bat) (S4) from Sekayu, Terengganu. All these five samples showed an expected RT-PCR product of 120 base pairs (bp) (Figure 2).

Sequence analysis

NCBI-BLAST results showed that based on partial 3' UTR sequences, all four viruses from Sekayu (S4, S7, S11 and S13) have 98% sequence identity with human SARS-CoV-2, Hu_US_Colo_2022 detected from Colorado, USA. While one sample from Gunung Reng, Kelantan

(R63) was closely related to human SARS-CoV-2 (Hu_US_Ari_2021) reported from Arizona, USA with 99% sequence identity (Table 3). Sequence alignment of the viruses showed there was an insertion of 25 nucleotides in the R63 virus (Figure 3). Based on these partial 3'UTR sequences, the four viruses from Sekayu Terengganu have 100% sequence similarity but only 95% sequence similarity with the virus from Gunung Reng, Kelantan (R63), indicating that Sekayu viruses are different from Gunung Reng virus (Table 3). Blast results of partial 3'UTR sequences of these viruses showed that viruses from Sekayu have 97% and 96% sequence identities with Hu_Wu_CoV_2019 and bat coronavirus BatCoV_RaTG13, respectively. Meanwhile, R63 with 99% sequence identity with Hu-Wu_CoV_2019 and BatCoV_RaTG13, respectively (Table 3). The sequences of these SARS-CoV-2-like coronaviruses were deposited in the NCBI-GenBank with these accession numbers: S4 (OQ946980), S7 (OQ135136), S11 (OQ135137), S13 (OQ135138) and R63 (OQ135139).

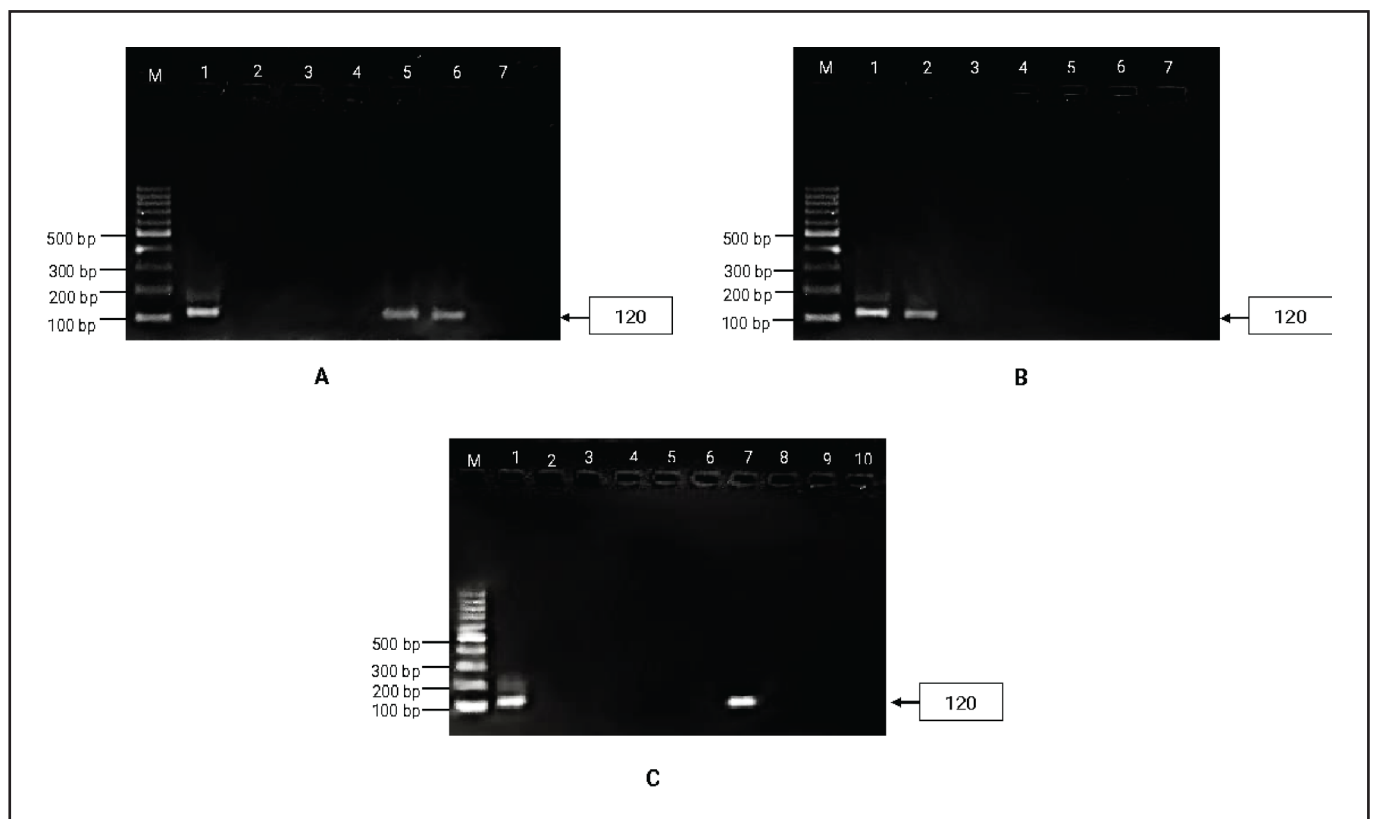


Figure 2. RT-PCR of representative bat samples. (A) and (B) were samples taken from Sekayu, Terengganu, meanwhile (C) are samples taken from Gunung Reng, Kelantan. (A) Lane 1 and 5 -Positive samples (S4 and S7); lane 2,3,4 - Negative samples; lane 6-Positive control and lane 7-Negative control. (B) Lane 1 and 2- Positive samples (S11 and S13); lane 3,4,5,6,7-Negative samples (C) Lane 1-Positive control; lane 2-Negative control; lane 3,4,5,6,8,9,10 - Negative samples; lane 7- R63 positive sample.

Table 3. Nucleotide sequence identities (%) of 3' UTR sequences between Bat coronaviruses from East Coast Malaysia and published sequences of coronavirus from the NCBI-Genbank.

	S4	S7	S11	S13	R63	Hu_US_Colo_2022	Hu_US_Ari_2021	Bat_CoV - RaTG13	Hu-Wu_2019	Pangolin_China 2019
S4	100	100	100	100	95	98	93	96	97	96
S7		100	100	100	95	98	93	96	97	96
S11			100	100	95	98	93	96	97	96
S13				100	95	98	93	96	97	96
R63					100	95	99	93	94	93
Hu_US Colo_2022						100	95	98	99	95
Hu_US_Ari_2021							100	95	95	95
Bat_CoV - RaTG13								100	99	98
Hu-Wu_2019									100	99
Pangolin_China 2019										100



Figure 3. Sequence alignment of 3' UTR region at the nucleotide position 44 to 164 of the bat coronaviruses from East Coast Malaysia.

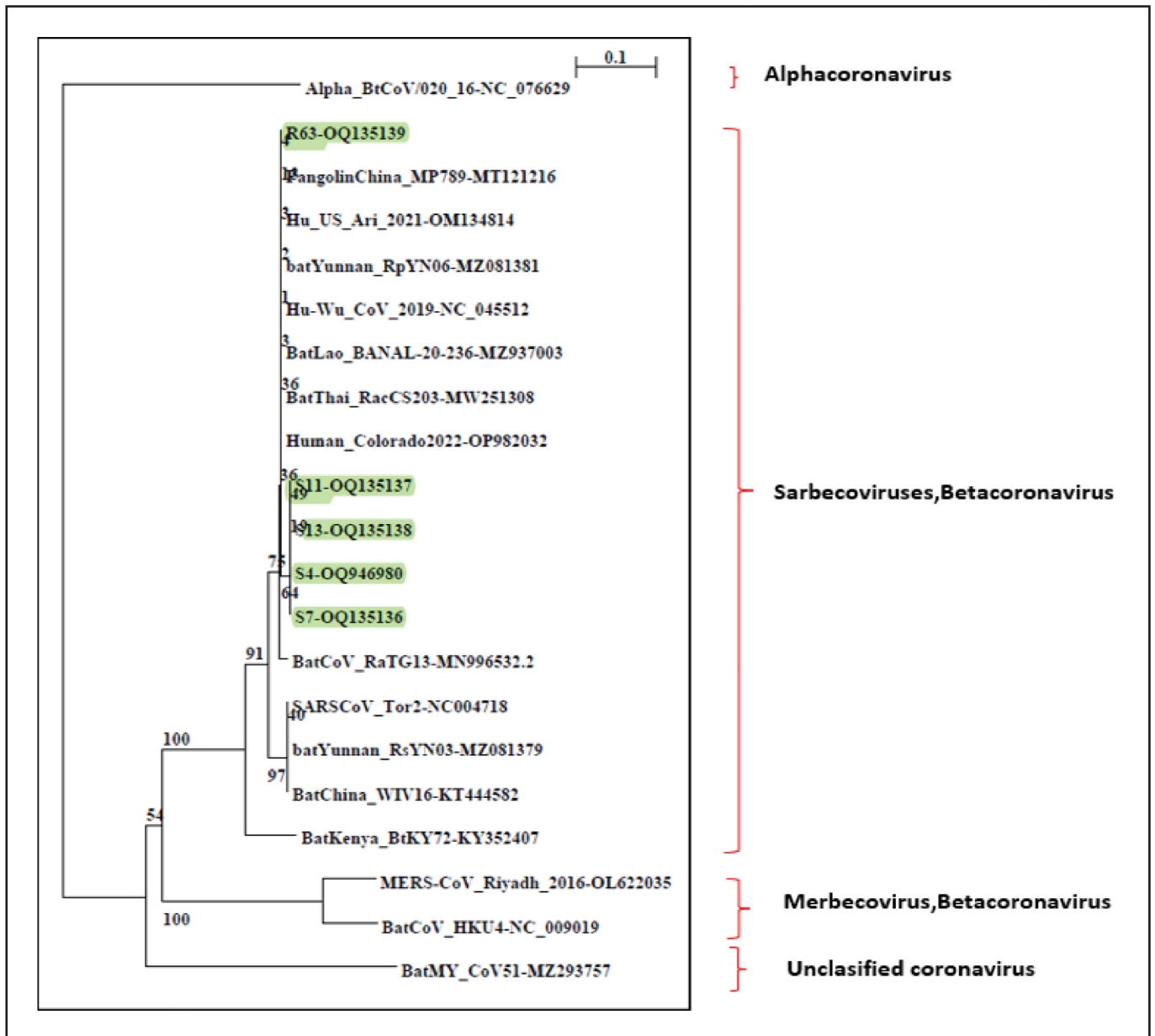


Figure 4. Phylogenetic analysis of SARS-CoV-2-like bat coronaviruses from East Coast, Malaysia (S4- OQ946980, S7-OQ135136, S11-OQ135137, S13-OQ135138 and R63-OQ135139-highlighted in green). The phylogenetic tree was constructed based on 120 base pairs of partial 3'UTR sequences. Reference sequences were retrieved from NCBI GenBank with written accession numbers at the back and aligned using ClustalX. The tree was constructed using the neighbor-joining (NJ) method with bootstrap values of 100 replicates with Alpha_BtCoV/020_16-NC_076629 sequence from bat Finland was used as an outgroup.

Phylogenetic Analysis

Phylogenetic tree based on a partial 3' UTR gene (Figure 4) showed that four viruses from Sekayu (S4, S7, S11 and S13) are clustered together in the same clade which was derived from a clade that contain R63 from Gunung Reng, human SARS-CoV-2 (Hu-Wu_CoV_2019, Hu_US_Ari_2021 and Hu_Colorado2022) and the bat SARS-CoV-2 like viruses from Asia (BatLao_BANAL-20-236, BatThai_RacCS203, BatYunnan_RpYN06,) and pangolin coronavirus from China (PangolinChina_MP789). All these viruses are derived from BatCoV_RaTG13. These viruses are in different lineages and distant from the SARS-CoV, MERS-CoV, BatKenya_BtKY72 and BatMY_CoV from Sabah, Malaysia. BatChina_WIV and batYunnan_RsYN03 are in clustered in the same clade as SARS-CoV, while batCoV_HKU4 in the MERS-CoV clade. These results are expected since these bat CoVs are believed to be an ancestor of SARS-CoV and MERS-CoV, respectively (Figure 4).

DISCUSSION

Many SARS-CoV-2-like viruses were detected in bats in South East Asia and speculated as an ancestor of SARS-CoV-2 (Delaune *et al.*, 2021, Mallapaty, 2021, Wacharapluesadee *et al.*, 2021). In this study, we have successfully detected five bat samples in East Coast Malaysia positive for SARS-CoV-2 like coronaviruses using our designed primers based on the 3' UTR gene. We failed to get a positive band with published primers based on the RdRp gene. This could be due to the sequence variation at the 3' end of the primers with the RNA sequences of the studied viruses. New primers based on the 3'UTR region were designed due to our previous experiment with infectious bronchitis virus (Avian coronavirus), where higher positive RT-PCR results were obtained using primers based on this gene compared to the other genes (Mohamed, 2000). Besides,

Chan *et al.* (2020) claimed that this gene is conserved and has a potential as a therapeutic target. To date, there is no report on the presence of public health importance coronaviruses such as MERS-CoV, SARS-CoV and SARS-CoV-2 from bats in Malaysia. Although Tan *et al.* (2021) have detected 10 coronaviruses from bats in Sarawak, Malaysia but their findings showed that the viruses were distant from the public health importance human coronaviruses. These Sarawak bat coronaviruses cannot be included in this study due to the unavailability of 3'UTR sequences in the NCBI GenBank. Only bat coronavirus from Sabah (BatMY_CoV) which was detected in 2012 was included and it was also distant from the MERS-CoV, SARS-CoV and SARS-CoV-2.

Based on sequence identities of whole genome of the viruses, the closest bat coronaviruses to human SARS-CoV-2 are strain RaTG (96.2%), strain RpYN06 (94.48%) and strain RmYN02 (93.3%) detected in *Rhinolopus affinis* (*R. affinis*), *R. pusillus* and *R. malayanus*, respectively, which were collected in Yunnan province of China (Liu *et al.*, 2021; Zhou *et al.*, 2020, 2021). In our study, out of five positive samples, two were detected from *Rhinolopus* spp. (also known as horseshoe bats), this is in agreement that *Rhinolopus* spp. are the main natural reservoir and source of zoonotic coronaviruses. Many studies have proposed that the ancestor of SARS-CoV originated from coronaviruses circulating in different horseshoe bat species (Alkhovsky *et al.*, 2022; Wacharapluesadee *et al.*, 2021). A study in Cambodia has shown that two of the positive samples (strains RshSTT182 and RshSTT200) were detected from *Rhinolopus shameli* (*R. shameli*) bat species (Delaune *et al.*, 2021). Other viruses that are closely related to SARS-CoV-2 are strains BANAL-52, BANAL-103 and BANAL-236, which were also detected from bat *R. malayanus*, *R. pusillus*, and *R. marshalli*, respectively from Laos (Mallapaty, 2021). This study also revealed that SARS-CoV-2-like viruses can be found in other species of bats (*Eonycteris*, *Balionycteris* and *hipposideros* spp). This supports the finding by Mendenhall *et al.* (2017), which also detected Betacoronavirus from *Eonycteris* feces and urine.

Phylogenetic analysis of 3'UTR gene of the studied coronaviruses revealed that bat coronaviruses from East Coast Malaysia are closely related to human SARS-CoV-2 and SARS-CoV-2 like viruses in bats from South East Asia (Thailand and Laos) and China which were derived from BatCoV_RaTG13. The finding of this study further confirmed that coronaviruses from bats in Southeast Asia are the hotspot of diversity for SARS-CoV-2-related viruses. The possibility of this virus being circulated from one site to another in a close geographical location is high since the migration of these bats is widespread and they often share the same roosting sites (Willoughby *et al.*, 2017). Thus, continuous surveillance of the presence of zoonotic-potential coronaviruses in bats and other wild animals in Southeast Asia is crucial for future pandemic preparedness and prevention.

CONCLUSION

This study revealed that SARS-CoV-2 like coronaviruses present in bats from East Coast Malaysia. This is the first report on the presence of SARS-CoV-2-like coronaviruses in bats from East Coast Malaysia that may give a pandemic-potential threat to humans in the future. Sequence analysis based on the partial 3'UTR region revealed that the viruses from Sekayu Terengganu (S4, S7, S11, S13) are slightly different from the virus from Gunung Reng, Kelantan (R63). These bat coronaviruses are closely related to Human SARS-CoV-2 and bat SARS-CoV-2 like viruses from Asia. However, sequence analysis was only targeted a short sequence of conserved 3'UTR. Thus, sequence analysis based on complete genome or spike of the viruses is crucial to get a better picture of the genetic variation of these viruses.

Besides, further study on the zoonotic-potential of these viruses by assessing the ability of the viruses to bind to the human host receptor and the ability of the virus in inhibiting the interferon production are needed for future preparedness of the pandemic.

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Conflict of Interest

All the authors declares that they have no conflict of interests.

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