Investigating the Presence of Key Amino Acid Residues for Human ACE-2 Receptor Binding Domain (RBD) of SARS-CoV-2-like Coronaviruses Isolated from Bats in East Coast Malaysia

ASHLEY JOYCE POON

FACULTY OF VETERINARY MEDICINE

2023

Investigating the Presence of Key Amino Acid Residues for Human ACE-2 Receptor Binding Domain (RBD) of SARS-CoV-2-like Coronaviruses Isolated from Bats in East Coast Malaysia

Ashley Joyce Poon

D19A0003

A Research Paper Submitted to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan in Partial Fulfilment of the Requirement for Degree of Doctor of Veterinary Medicine

Faculty of Veterinary Medicine

UNIVERSITI MALAYSIA KELANTAN

2023

INVESTIGATING THE PRESENCE OF KEY AMINO ACID RESIDUES FOR HUMAN ACE-2 RECEPTOR BINDING DOMAIN (RBD) OF SARS-COV-2-LIKE CORONAVIRUSES ISOLATED FROM BATS IN EAST COAST MALAYSIA

ABSTRACT

An abstract of the research paper submitted to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial fulfilment of the course DVT55204 – Research Project.

Covid-19 is a recently emerging form of severe acute respiratory syndrome (SARS) caused by SARS-Coronavirus-2 (SARS-CoV-2) which was first reported in Wuhan, China in December 2019. Bats are natural reservoirs for coronaviruses, and bat coronaviruses have been highlighted as some strains of SARS-like coronavirus (SARSL-CoV-2) carried by bats are almost identical to human SARS-CoV-2 at the genome level, especially the spike protein. Some strains of these bat coronaviruses are highly similar to human SARS-CoV-2 and are able to use the same receptor as SARS-CoV-2 to mediate cell entry. Early last year, five SARS-CoV-2-like viruses were isolated from bats in East Coast Malaysia (four from Sekayu, Terengganu and one from Gunung Reng, Kelantan). Angiotensin-converting enzyme 2 (ACE-2) has been identified as the important receptor of SARS-CoV and SARS-CoV-2 to enter human cells. The interaction between the S1 protein of the virus, particularly amino acid fragment 318-510, with the human ACE-2 receptor is the critical determinant of coronavirus host range and cross-species transmission. This study aimed to investigate the presence of key amino acid residues for human ACE-2 on the RBD of the spike protein of these bat coronaviruses. Reverse transcription polymerase chain reaction (RT-PCR), and sequencing of the PCR products were used to investigate the presence of the ACE-2 RBD. From the results obtained in this experiment, it was determined that coronaviruses isolated from bats in East Coast Malaysia can utilise human ACE-2 receptors to infect human cells and thus may become a public health concern in the future.

Keywords: SARS-CoV-2-like coronaviruses, ACE-2 receptors, Receptor Binding Domain (RBD), bat coronaviruses, human transmission

ABSTRAK

Abstrak kertas penyelidikan yang dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelatan untuk memenuhi sebahagian dari syarat keperluan kursus DVT55204 – Projek Penyelidikan.

Covid-19 merupakan suatu bentuk penyakit sindrom pernafasan akut baru yang parah yang disebabkan oleh SARS-Coronavirus-2 (SARS-CoV-2) yang pertama kalinya dilaporkan di Wuhan, China pada Disember 2019. Kelawar merupakan takungan semula jadi untuk virus ini, dan lebih penekanan telah diberikan pada virus korona kelawar. Hal ini kerana beberapa strain *SARS-like coronaviruses* (SARSL-CoV) yang dikesan pada kelawar hampir serupa dengan SARS-CoV-2 pada manusia pada peringkat genomik, terutatamanya pada protein spike. Setengah strain virus korona dari kelawar ini sangat serupa dengan SARS-CoV-2 manusia dan boleh menggunakan reseptor enzim penukar angiotensin 2 (ACE-2) manusia sebagai pengantaraan untuk memasuki sel manusia Pada awal tahun lalu, lima virus korona yang menyerupai SARS-CoV-2 dari Pantai Timur Malaysia (empat dari Sekayu, Terengganu dan satu dari Gunung Reng, Kelantan) telah diisolasi dari kelawar. Reseptor enzim penukar angiotensin 2 (ACE-2) telah ditakrifkan sebagai reseptor penting untuk-SARS-CoV dan SARS-CoV-2 menembusi sel manusia. Interaksi antara protein S1 virus, terutamanya residu asid amino 318-510 dengan reseptor ACE-2 manusia merupakan penentu kritikal untuk tropisme tisu tertentu dan julat perumah koronavirus. Kajian ini bertujuan untuk menyiasat kewujudan residu amino asid yang kritikal untuk domain pengikat reseptor enzim penukar angiotensin 2 dalam virus korona kelawar tersebut. Reverse transcription polymerase reaction (RT-PCR), dan jujukan asid amino telah digunakan untuk menyiasat kehadiran RBD untuk ACE-2 virus tersebut. Hasil keputusan eksperimen ini telah mengesahkan bahawa virus korona yang diisolasi dari kelawar di Pantai Timur Malaysia berkeupayaan menjangkiti sel manusia dan berkemungkinan menjadi isu kesihatan awam pada masa yang akan datang.

Kata kunci: *SARS-like coronaviruses,* reseptor enzim penukar angiotensin 2, domain pengikat reseptor, koronavirus kelawar, penularan kepada manusia

ORGINAL LITERARY WORK DECLARATION

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

☐	**OPEN ACCESS**	I agree that my thesis is to be made immediately available as hardcopy or online open access (full text).
☐	**EMBARGOES**	I agree that my thesis is to be made available as hardcopy or online (full text) for a period approved by the Post Graduate Committee. Dated from _____ until _____.
☐	**CONFIDENTIAL**	(Contains confidential information under the Official Secret Act 1972) *
☐	**RESTRICTED**	(Contains restricted information as specified by the organisation where research was done) *

I acknowledge that Universiti Malaysia Kelantan reserves the right as follows.

1. The thesis is the property of Universiti Malaysia Kelantan.

2. The library of Universiti Malaysia Kelantan has the right to make copies for the purpose of research only.

3. The library has the right to make copies of the thesis for academic exchange.

Ashley Joyce _____

SIGNATURE OF CANDIDATE SIGNATURE OF SUPERVISOR

000907-13-1114 _____

NRIC/PASSPORT NO. NAME OF SUPERVISOR

DATE: 28 February 2024 DATE:

AKNOWLEDGEMENTS

All gratitude and honour to God Almighty, for through Him I can do all things.

Isaac Newton once said, *"If I have seen further, it is by standing on the shoulders of Giants."* Therefore, I thank my supervisors, Prof. Dr. Maizan binti Mohamed and Prof. Madya Dr. Sandie Choong Siew Shean for their knowledge and insight throughout this project, which has made this project so much more fulfilling and interesting for me. My sincerest appreciation also goes to Miss Nani Izreen, for her patience with my inexperience in the lab and her guidance.

To my parents, without whom I would not even be in vet school, much less survive to my final year. And to my brother, Aaron, who taught me all the good things I know. To Elsa Cordelia, the older sister I never had. Thank you for listening.

To Dr. Ashyer Andderias, my best friend and my constant. Thank you for sharing both my good and bad days. Your encouragement means the world to me.

To Cookie, Mitten, Trio, and my little slipper thief; the thought of you keeps me going.

I humbly dedicate this to you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

SARS	-	Severe acute respiratory syndrome
SARS-CoV	-	Severe acute respiratory syndrome coronavirus
MERS-CoV	-	Middle East respiratory syndrome coronavirus
COVID-19	-	Coronavirus disease 2019
SARSL-CoV	-	Severe-acute-respiratory-syndrome-like coronavirus
ACE2	-	Angiotensin-converting enzyme 2
RNA	-	Ribonucleic acid
RT-PCR	-	Reverse transcription polymerase chain reaction
RBD	-	Receptor binding domain
SARS-CoV-2	-	Severe acute respiratory syndrome coronavirus 2
CoV	-	Coronavirus
ORF	-	Open reading frame
RTC	-	Replication and translation complex
VoC	-	Variants of concern
ATCC	-	American type cell culture
TBE	-	Tris-borate-EDTA
CPE	-	Cytopathic effect

CHAPTER 1
INTRODUCTION

1.1 Introduction

SARS-associated coronavirus causes a viral respiratory disease termed severe acute respiratory syndrome (SARS). Covid-19 is an emerging form of SARS caused by SARS-coronavirus-2 (SARS-CoV-2) and it was first reported in Wuhan, China in December 2019 (Zhu N *et al.*, 2020). Covid-19 spreads by airborne transmission through respiratory aerosols and by direct contact with contaminated surfaces, known which is also known as fomites (Chia C. Wang *et al.*, 2021).

Since bats are natural reservoirs for coronaviruses, there have been concerns regarding whether bats are able to transmit this respiratory disease to humans. There has been more emphasis placed on bat coronaviruses as they have caused three novel disease outbreaks in humans, namely SARS-CoV, Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and Coronavirus disease 19 (COVID-19). The spike protein of some strains of SARS-like coronavirus (SARSL-CoV) carried by bats are almost identical to SARS-CoV, SARS-CoV-2 and MERS-CoV, especially at the genome level of the spike protein (Hu *et al*., 2015). They are also known to use the same angiotensin converting enzyme-2 (ACE-2) receptors to mediate entry into host cells (Hu *et al.*, 2015). This relates to the primary concern of this study, since SARS-CoV-2-like coronavirus have previously been isolated from bats in East Coast Malaysia, it is vital for us to determine whether these SARS2L-CoV can utilise human ACE-2 receptors to mediate entry into human cells as this might pose a public health concern in the future.

In this experiment, we investigate the presence of amino acids critical for human ACE-2 receptor binding in the receptor binding domain of SARS-CoV-2-like coronaviruses isolated from bats in East Coast Malaysia to determine the zoonotic

potential of the virus. Since SARS-CoV-2 is an RNA virus, we used reverse transcription polymerase chain reaction (RT-PCR) to amplify the spike gene of the viruses in where the receptor binding domain (RBD) is located. The PCR product was observed for the presence of the expected band using gel electrophoresis and sequenced to determine the presence of critical amino acids for human ACE-2 receptor binding domain of the viruses.

1.2 Problem Statement

Bats are known as reservoirs for coronaviruses. Despite this, not many studies have been conducted to determine the transmission of the virus from bats to humans. Therefore, this study aims to detect the presence of amino acids critical for human ACE-2 receptor binding on the receptor binding domain of SARS2L-CoV isolated from bats in East Coast Malaysia to determine the possible zoonotic potential.

1.3 Research Question

Are amino acids critical to human ACE-2 receptor binding present in SARSL-CoV isolated from bats in East Coast Malaysia?

1.4 Research Hypothesis

Amino acids critical for the binding of human ACE-2 receptors are present in SARSL-CoV isolated from bats in East Coast Malaysia.

1.5 Research Objectives

1. To detect the presence of amino acids critical for binding of human ACE-2 receptors in SARSL-CoV isolated in bats from East Coast Malaysia.
2. To determine the possible zoonotic potential of SARSL-CoV isolated in bats from East Coast Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Morphology and characteristics of SARS-CoV-2

SARS-Cov-2 is one of the viruses from the *Coronaviridae* family and order *Nidovirales* (Grudlewska-Buda et al., 2021). They are present in oval to pleomorphic forms. A single viral virion is about 50-200 nm in size (Finlay *et al*., 2004). Coronaviruses have one of the largest genomes (26-32 kb) amongst all the RNA viruses which is flanked by 3' and 5' untranslated regions containing RNA structures for RNA synthesis. The 5' end has two open reading frames (ORF), which are ORF-1a and ORF-1b. These two ORFs encode for non-structural proteins, which mostly comprise the viral replication and translation complex (RTC). Meanwhile, the ORF at the 3' end encodes structural and accessory proteins (V'kovski *et al.*, 2020). The viral envelope is made of three proteins: the S-spike protein, the M-membrane protein, and the E-envelope protein (Figure 1). Meanwhile, the structural component of the nucleocapsid is formed by the N-nucleocapsid protein while the RBD is located in the S1 gene of CoVs (Grudlewska-Buda *et al*., 2021).

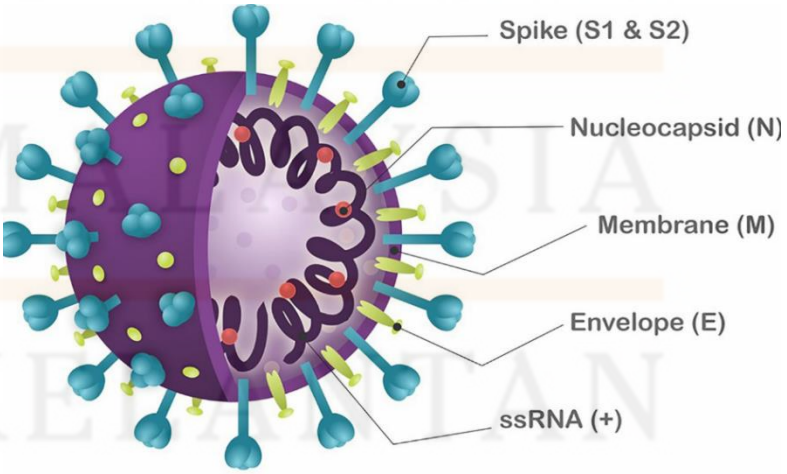

Figure 1. Structure of Coronavirus (Priyadarshi *et al.*, Coatings, 2022)

2.2 Bats as reservoirs of important public health viruses

It has been well known for quite some time that bats are the reservoirs for many human diseases. However, bats have again reclaimed the attention of researchers as they are the natural reservoirs of multiple severe zoonotic diseases such as Nipah virus, Hendra virus, Ebola virus, Marburg virus, and most recently, SARS-CoV-2, to name a few (Han *et al.,* 2015). Multiple studies have been conducted to truly understand how viruses circulate and maintain persistence in bat populations. One such study speculated that oscillating herd immunity is the primary reason behind persistently infected bat population, together with the connectivity between bats and their population size (Letko *et al.*, 2020). Despite proof that Nipah and Marburg viruses can replicate and shed in bats, these bats have displayed no obvious pathologies in response to the presence of these viruses in their bodies. Current studies suggest that the absence of typical lesions in bats may be due to the absence of strong immune response from the bats compared to human and lab animal models (Letko *et al.,* 2020). Thus, the ability of viruses to remain persistent in bat populations coupled with the lack of common clinical signs may be the reason for efficient maintenance and dissemination of such viruses. Despite the rarity of close contact between humans and bats, spillover of these viruses may occur between bats and livestock, resulting in human infection. As an example, Nipah virus was accidentally introduced to livestock pig populations by contamination of pig swill with bat guano (Looi and Chua, 2007). Wildlife-human conflicts such as encroachment of humans into bat habitats may also have led to exposure to these viruses (Han *et al.,* 2015). In addition, bats can inhabit diverse ecological distribution (except for Antarctica), thus bringing them into close contact with

humans, resulting in a public health threat to the general human population (Han *et al*., 2015).

2.3 Bat origins of human coronaviruses

In recent years, many zoonotic coronaviruses have been found in various species of bats (de Groot *et al.*, 2012). 7 viral species of the genera Alphacoronavirus and Betacoronavirus, which mainly affect mammals, have been identified solely in bats. In 2005, SARSL-CoV Rp3, a type of SARSL-CoV, was discovered in horseshoe bats of the Rhinolophus genus in China. This SARSL-CoV displayed up to 87-92% resemblance to human SARS-CoV isolates at the full genome sequences (Li, 2005). However, there has been insufficient data about the human-animal interface for transmission of SARS-Cov-2 between bats and humans, and whether an intermediate host is involved (Liu Y-C *et al.*, 2020). Pangolins have been suspected as intermediate hosts for coronavirus transmission between bats and humans as they display a basal phylogenetic position relative to bat coronaviruses. Since bats and pangolins inhabit the same habitat, it is believed that bat coronaviruses may have undergone recombination in pangolins, thus developing an increased affinity for human ACE-2 receptors before spillover to humans (Temmam *et al.*, 2022).

2.4 Bat coronaviruses closely related to SARS-CoV, MERS-CoV, and SARS-CoV-2

SARS-CoV, MERS-CoV, and SARS-CoV-2 are three examples of recently emerging zoonotic coronaviruses. Viral isolates from bats have shown high sequence similarities to these coronaviruses. For example, coronaviruses

isolated from rhinolophid bats have shown 92% and 96% sequence identity similarity to SARS-CoV and SARS-CoV-2, respectively (Zhou *et al.,* 2020). Likewise, coronaviruses isolated from vespertilionid bats have shown a sequence identity of up to 86.5% with MERS-CoV, and related coronaviruses have also been found in different families of bats in Africa, North America, Europe, and Asia (Ruiz-Aravena *et al.,* 2021). Despite the resemblance between SARS related coronarviruses (SARSr-CoV) isolated from bats and those found in humans, none of the bat isolates match the human strain identically. The entire SARS-CoV genome has been sequenced across several individual but related viruses circulating in bat populations which strongly implies that the human strain of CoVs is a recombinant manifestation of these ancestral variants (Letko *et al.,* 2020).

2.5 Role of spike protein and receptor binding domain (RBD) in human host cell entry

The surface glycoprotein of SARS-CoV-2 contains two protein subunits, namely S1 and S2. S1 is the main subunit involved in recognising and binding to the host cell receptor. A 193 amino acid fragment (amino acid 318 – 510) on S1 is shown to be more efficient in binding to the human angiotensin-converting enzyme 2 (ACE-2) determining it to be the receptor binding domain (RBD) of SARS-CoV-2 (Hu *et al.,* 2015). During host cell attachment, the S1 subunit binds to the ACE-2 while the S2 subunit docks the S protein to the viral particle and mediates membrane fusion with host cell (Jackson *et al.,* 2021). S protein association with the ACE-2 receptor reveals the S2' site, which is an additional site within the S2 subunit. Transmembrane protein serine 2 (TMPRSS2) cleaves

the S2' site at the cell surface to release a fusion peptide. This fusion peptide is critical to form a fusion pore, permitting the viral genome to reach the host cell cytoplasm (Jackson *et al.,* 2021).

2.6 Receptor binding domain (RBD) mutations in bat coronaviruses that modulate binding to human ACE-2

Since the emergence of SARS-CoV-2 in humans, its fitness has been influenced by several sporadic mutations and recombination events, allowing the virus to spread extensively by evading neutralisation by host antibodies. The importance of the spike protein has frequently been highlighted since it determines the RBDS's affinity and accessibility to ACE-2 receptors. Hence, it is mostly responsible for the viruses' host range (Temmam *et al.,* 2022). A loop subdomain (amino acid 424 – 494) within the RBD contacts the ACE-2 receptor directly and has been acknowledged as the receptor binding motif (RBM). Several amino acid residues in this RBM were found to be critical for receptor binding and alterations in these amino acid residues were found to affect the binding efficiency in different SARS-CoV isolates (Li *et al*., 2005) and SARS-CoV-2 viruses (Hoffmann *et al.,* 2020). The presence of amino acid residues N426, Y436, Y440, L442, S456, F472, N473, Q479 at the RBD of S1 are believed to be involved in the ACE-2 binding of human cells (Hoffmann *et al.,* 2020; Zhou *et al*., 2021) as shown in Figures 2 and 3 respectively, with L442, F472, Q479, S480, N487 and Y491 noted as important key residues for binding to human ACE-2 receptors (Zhou *et al.,* 2021).

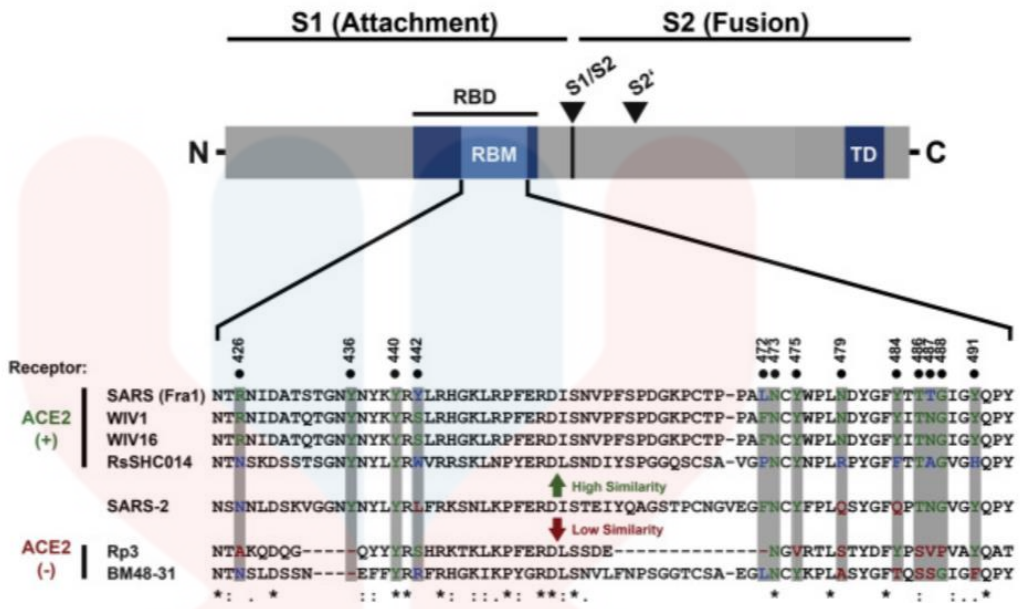

Figure 2. Highlighted amino acid residues believed to be involved in binding with human ACE-2 receptors (Hoffman et al., 2020)

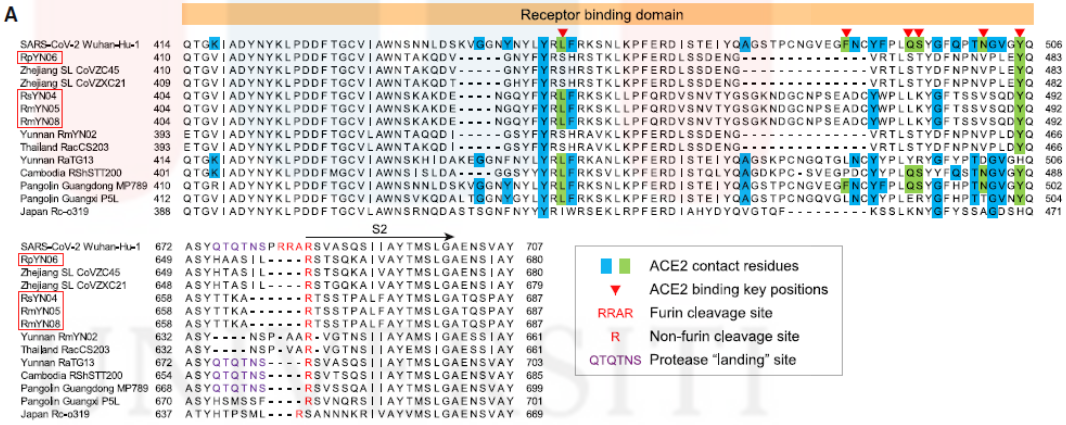

Figure 3. Important key residues at positions L442, F472, Q479, S480, N487 and Y491 shown by the red arrowheads (Zhou et al., 2021)

Therefore, for the bat coronaviruses to infect human, it is essential for them to have these amino acid residues at their S1 RBD. Despite bearing a 96% genome sequence similarity to SARS-CoV-2, RaTG13, a bat virus, is not shown to interact efficiently interact with human ACE-2 receptors. However, Zech *et al.* (2021) had demonstrated that a single mutation to the RaTG13 spike protein

T403R had massively increased its affinity to human ACE-2 receptors, which suggests that mutations of the spike protein may have aided in species crossover of coronaviruses, resulting in a zoonotic potential (Zech *et al.*, 2021). Moreover, Gupta *el al.* (2022) suggested that differences in SARS-CoV-2 spike proteins sequences, efficiency of ACE-2 usage, and pathophysiology of bats and humans implies the circulation and mutation of progenitor virus in an intermediate host before crossover to humans. From their studies, they have found that Pangolin-CoV isolated in 2019 from Guandong, China required a single semi-conservative mutation for human receptor binding, which strongly implied that GD Pangolin-CoV was involved in the origins of SARS-CoV-2 (Gupta *et al.*, 2022).

CHAPTER 3

MATERIALS AND METHODS

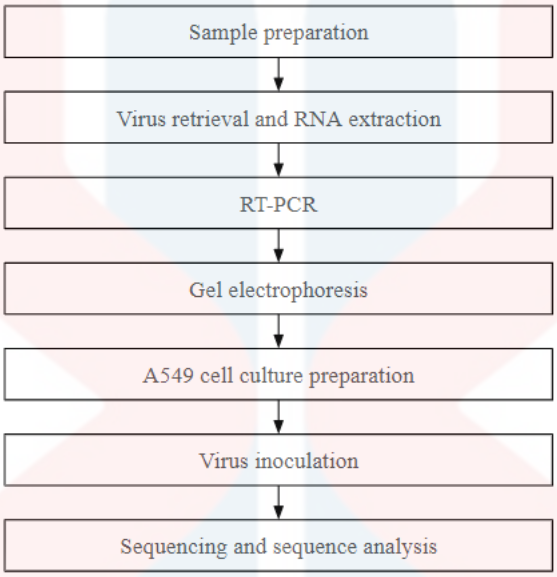

Figure 4. Flowchart of the study

4.1 Sample preparation

Samples for this experiment were obtained from the archives of the Virology Laboratory of the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. These samples were obtained from five bats from East Coast Malaysia, where one sample was from Gunung Reng, Kelantan and four samples from Sekayu, Terengganu, confirmed positive for coronaviruses from oropharyngeal swabs using RT-PCR on 3' untranslated region. The samples were passaged once in A549 human lung cells and kept in -70^{o}C freezer.

4.2 Virus retrieval and RNA extraction

Prior to RNA extraction, the virus was first retrieved from A549 human lung cells kept in -70^{o}C freezer. To release the virus from the cells, the cells were subjected to three freeze-

thaw cycles for cell lysis. RNA extraction was then carried out using a commercial RNA extraction kit, GENEzol (GeneAids, Taiwan) according to the manufacturer's protocol. First, 250 µL of the sample was mixed with 750 µL of GeneZOL. This was stored in room temperature for five minutes to allow complete dissociation of the nucleoprotein complexes. Then, 200 µL of chloroform was added and the mixture was shaken vigorously for 15 seconds. The mixture was then centrifuged at 14000 g for 15 minutes at 4°C. The aqueous phase of the solution was transferred into a new sterile 1.5 mL microtube. Next, 500 µL of isopropanol was added and the mixture was rested at room temperature for ten minutes. Following that, the mixture was centrifuged again at 14000 g for ten minutes at 4°C. After centrifugation, the RNA precipitated and formed a gel-like white pellet on the bottom of the tube. The supernatant was removed and one mL of cold 75% ethanol was added to the pellet. This was shaken vigorously for 15 seconds and centrifuged again at 14000 g for five minutes at 4°C. The supernatant was removed, and RNA was seen as a white pellet. The pellet was airdried for 15 minutes. Once dry, the pellet was dissolved in 30 µL nuclease free water and mixed well by pipetting up and down. The mixture was incubated at 60°C for five minutes and stored at -80°C until ready for RT-PCR.

4.3 Reverse transcription polymerase chain reaction (RT-PCR)

Following the RNA extraction, RT-PCR was carried out using primers previously designed by our group that flank the RBD on S1 gene of SARS-CoV-2. These primers were designed manually based on sequences alignment of S1 genes of bat and human coronaviruses retrieved from National Centre for Biotechnology Information (NCBI) GenBank. The sequences of the primers are 950RBDF 5' CTAACTTTAGAGTCCAACC 3' and 1623RBDR 5' CACCTGTGCCTTTTAAACC 3' which produced a 509 bp RT-PCR product. To create the

Mastermix, 25 µL of 2X AccessQuick (Promega Corporation, USA), one µL of AMV-RT (Promega Corporation, USA), two µL of ten µM forward and reverse primers respectively, 2.8 µL of RNAsin, 14.6 µL of nuclease free water, and five µL of viral RNA sample. Mixing was conducted on ice to prevent denaturation of RNA and enzymes used. The conditions for RT-PCR cycle were set at 42°C for 60 min, inactivation of the reverse-transcriptase enzyme at 94°C for 2 min, followed by 35 cycles of PCR amplification at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec and one final extension cycle at 72°C for 7 min. The reaction was conducted in a thermocycler (Biorad T100, USA).

4.4 Gel electrophoresis

PCR products were analysed in 1.5% agarose gel. The agarose gel was prepared by weighing 1.2g of agarose powder (Vivantis) and dissolving it with 80 mL of 1X Tris-Borate-EDTA (TBE) buffer in a conical flask before heating it to dissolve the agarose powder. One µL of Midori Green dye (Nippon Genetics, Europe) was added to the mixture before pouring it into the pre-casted tray. Once the gel was solidified, the comb was removed with caution. Ten µL of PCR sample was then loaded into each well, while five µL of 100 bp DNA marker was loaded into the center well. The products were run at 100 V for 30 minutes and visualised using Gel DocTM EZ Gel Imager (Bio-Rad, USA).

4.5 A549 cell culture preparation

For A549 cell culture preparation, the lung cells in the cryovial tube were thawed at 37^{o}C in shaking bath water. In the meantime, five mL of 10% fetal bovine serum (FBS) Dulbecco's Modified Eagle Serum (DMEM) with 1% Penicillin-streptomycin was prewarmed at 37^{o}C. Once warm, four mL of the 10% FBS DMEM media was transferred into a T25 flask

together with one mL of thawed cell suspension. The cell suspension was then incubated in 5% CO_2 at 37°C and observed with daily media changes to prevent cell debris buildup until 75% confluency was reached. When 75% confluency was confirmed by viewing under inverted microscope, passaging of cells was carried out. 10% FBS DMEM, phosphate buffered saline (PBS), and trypsin ethylenediaminetetraacetic acid (trypsin-EDTA) were prewarmed to 37 ^oC. The cell suspension was washed with PBS twice; the flask was gently rocked and inverted. The PBS was then removed from the flask. Then, two mL of trypsin-EDTA was added until the bottom of the culture vessel was evenly covered. The cells were incubated for 5 minutes to allow full detachment. After incubation, the flask was tapped firmly to detach the cells, and detachment was confirmed by viewing under an inverted microscope. Three mL of 2% FBS DMEM was added to the flask to halt trypsin-EDTA activity. The mixture was aspirated to prevent clumping. Then, five mL of cell suspension was transferred into a new flask with 10 mL of 10% FBS DMEM. After 75% confluency was observed in new flask, the process was repeated for the third and final passage.

4.6 Virus inoculation

Two mL of the cells (10^5 cells/mL) were seeded in each well of a 6-well plate. The 6-well plate was then incubated in 5% CO_2 at 37°C until 75% confluency was achieved. Then, the cells were washed twice to remove cellular debris buildup. After washing, 1.6 mL of 2% FBS media was added to each well and 400 µL of passage two bat virus suspension was inoculated in 5 wells, while one well was served as a positive control with inoculation of human SARS-CoV-2 virus. The uninoculated cells in the T25 flask was used as negative control. The 6-well plate was incubated for 7 days at 37^oC in 5% CO_2 and checked daily until cytopathic effect (CPE) was observed.

4.7 Sequencing and sequence analysis

When RT-PCR product was confirmed by gel electrophoresis, the band was cut and sent to Apical Scientific Sdn. Bhd. Malaysia for sequencing in both forward and reverse directions. The obtained nucleotide sequences were edited using Bioedit 7.2 software (Informer Technologies, Inc.), converted to amino acid sequences using Generunner 6.5 software (Informer Technologies, Inc.), amino acid sequences were aligned using ClustalW (GenomeNet, Kyoto University Bioinformatic Center) and analysed manually for the presence of important amino acids at certain positions that are critical for human ACE2 binding. The amino acids we are interested in are as follows: N426, Y436, Y440, L442, S456, F472, N473, S479 which are important in ACE-2 binding to human cells (Hoffmann *et al.*, 2020; Zhou *et al.*, 2021). The one-letter abbreviations are tabulated as in Table 1:

One-letter abbreviation	Amino acid
Y	Tyrosine
L	Leucine
N	Phenylalanine
F	Asparagine
Q	Glutamine
D	Aspartic acid
S	Serine

Table 1. Important amino acids for ACE-2 binding on S1 RBD of the viruses and their one letter abbreviations.

CHAPTER 4

RESULTS

5.1 RT-PCR product and gel electrophoresis

All five samples tested positive for the receptor binding domain (RBD) of SARS-CoV-2-like coronaviruses in this experiment using 950RBDF and 1623RBDR primers during RT-PCR. Gel electrophoresis of the RT-PCR samples was done to demonstrate the presence of RBD in the samples. Based on Figure 5, bands were observed for all viral samples and human SARS-CoV-2 positive control with the expected band size of 509 bp.

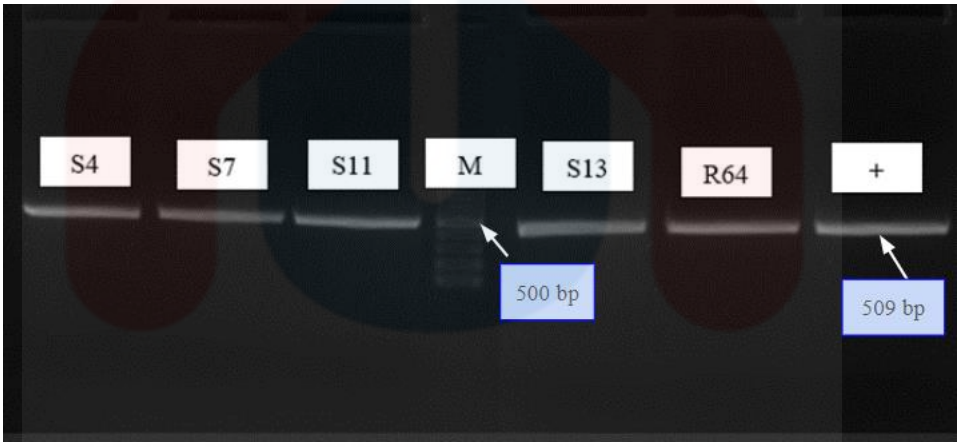

Figure 5. Gel electrophoresis of virus samples.

S4: SARS-CoV-2L sample from Sekayu, Terengganu

S7: SARS-CoV-2L sample from Sekayu, Terengganu

S11: SARS-CoV-2L sample from Sekayu, Terengganu

S13: SARS-CoV-2L sample from Sekayu, Terengganu

R64: SARS-CoV-2L sample from Gunung Reng, Kelantan

M: DNA ladder

+: Positive control

5.2 Sequencing result for nucleotide and amino acid sequences

Once the expected band was observed, the gel band was cut and sent to Apical Scientific Sdn. Bhd. Malaysia for sequencing in both forward and reverse directions along with the positive control. The coronavirus isolates sent for sequencing are listed as per Table 2., with human SARS-CoV-2 (HuSARS-CoV-2) obtained from Hospital Raja Perempuan Zainab 2 (HRPZ2) used as a positive control in this study.

Species	Coronavirus	Isolates	Year
Human	SARS-CoV-2	HuSARS-CoV-2	2019
		S4	2022
		S7	2022
Bat	SARS-CoV-2-L	S11	2022
		S13	2022
		R64	2022

Table 2. List of representative human and bat coronaviruses used for sequence alignment.

The nucleotide sequences were edited with Bioedit 7.2 software (Informer Technologies, Inc.) and converted into amino acid sequences using Generunner 6.5 software (Informer Technologies, Inc.). The nucleotide and amino acid sequences are tabulated in Table 3.

Virus isolate	Nucleotide sequence	Amino acid sequence
HuSARS-CoV-2	GTTTATGCTTGGAACAGGAAGAGAA TCAGCAACTGTGTTGCTGATTATTCT GTCCTATATAATTCCGCATCATTTTC CACTTTTAAGTGTTATGGAGTGTCTC CTACTAAATTAAATGATCTCTGCTTT ACTAATGTCTATGCAGATTCATTTGT AATTAGAGGTGATGAAGTCAGACAA ATCGCTCCAGGGCAAACTGGAAATA	VYAWNRKRISNCVADYS VLYNSASFSTFKCYGVSPT KLNDLCFTNVYADSFVIR GDEVRQIAPGQTGNIADY NYKLPDDFTGCVIAWNSN NLDSKVGGNYNYLYRLFR KSNLKPFERDISTEIYQAG STPCNGVKGFNCYFPLRS YSFRPV

	TTGCTGATTATAATTATAAATTACCA GATGATTTTACAGGCTGCGTTATAGC TTGGAATTCTAACAATCTTGATTCTA AGGTTGGTGGTAATTATAATTACCTG TATAGATTGTTTAGGAAGTCTAATCT CAAACCTTTTGAGAGAGATATTTCAA CTGAAATCTATCAGGCCGGTAGCAC ACCTTGTAATGGTGTTAAAGGTTTTA ATTGTTACTTTCCTTTACGATCATAT AGTTTCCGACCCAA	
S4	GAGAATCAGCAACTGTGTTGCTGATT ATTCTGTCCTATATAATTCCGCATCA TTTTCCACTTTTAAGTGTTATGGAGT GTCTCCTACTAAATTAAATGATCTCT GCTTTACTAATGTCTATGCAGATTCA TTTGTAATTAGAGGTGATGAAGTCA GACAAATCGCTCCAGGGCAAACTGG AAATATTGCTGATTATAACTATAAAT TACCAGATGATTTTACAGGCTGCGTT ATAGCTTGGAATTCTAACAATCTTGA TTCTAAGGTTGGTGGTAATTATAATT ACCTGTATAGATTGTTTAGGAAGTCT AATCTCAAACCTTTTGAGAGAGATAT TTCAACTGAAATCTATCAGGCCGGTA GCACACCTTGTAATGGTGTTAAAGGT TTTAATTGTTACTTTCCTTTACGATCA TATAGTTCCCGACCCAA	RISNCVADYSVLYNSASFS TFKCYGVSPTKLNDLCFT NVYADSFVIRGDEVRQIAP GQTGNIADYNYKLPDDFT GCVIAWNSNNLDSKVGG NYNYLYRLFRKSNLKPFE RDISTEIYQAGSTPCNGVK GFNCYFPLRSYSSRPR
S7	TCTGTTTATGCTTGGAACAGGAAGA GAATCAGCAACTGTGTTGCTGATTAT TCTGTCCTATATAATTCCGCATCATT TTCCACTTTTAAGTGTTATGGAGTGT CTCCTACTAAATTAAATGATCTCTGC TTTACTAATGTCTATGCAGATTCATT TGTAATTAGAGGTGATGAAGTCAGA CAAATCGCTCCAGGGCAAACTGGAA ATATTGCTGATTATAATTATAAATTA CCAGATGATTTTACAGGCTGCGTTAT AGCTTGGAATTCTAACAATCTTGATT CTAAGGTTGGTGGTAATTATAATTAC CTGTATAGATTGTTTAGGAAGTCTAA TCTCAAACCTTTTGAGAGAGATATTT CAACTGAAATCTATCAGGCCGGTAG CACACCTTGTAATGGTGTTAAAGGTT TTAATTGTTACTTTCCTTTACGATCAT ATAGTTTCCGACCCAA	SVYAWNRKRISNCVADYS VLYNSASFSTFKCYGVSPT KLNDLCFTNVYADSFVIR GDEVRQIAPGQTGNIADY NYKLPDDFTGCVIAWNSN NLDSKVGGNYNYLYRLFR KSNLKPFERDISTEIYQAG STPCNGVKGFNCYFPLRS YSSRPR
S11	CTGTTTATGCTTGGAACAGGAAGAG AATCAGCAACTGTGTTGCTGATTATT CTGTCCTATATAATTCCGCATCATTT TCCACTTTTAAGTGTTATGGAGTGTC	VYAWNRKRISNCVADYS VLYNSASFSTFKCYGVSPT KLNDLCFTNVYADSFVIR GDEVRQIAPGQTGNIADY

	TCCTACTAAATTAAATGATCTCTGCT TTACTAATGTCTATGCAGATTCATTT GTAATTAGAGGTGATGAAGTCAGAC AAATCGCTCCAGGGCAAACTGGAAA TATTGCTGATTATAATTATAAATTAC CAGATGATTTTACAGGCTGCGTTATA GCTTGGAATTCTAACAATCTTGATTC TAAGGTTGGTGGTAATTATAATTACC TGTATAGATTGTTTAGGAAGTCTAAT CTCAAACCTTTTGAGAGAGATATTTC AACTGAAATCTATCAGGCCGGTAGC ACACCTTGTAATGGTGTTAAAGGTTT TAATTGTTACTTTCCTTTACGATCAT ATAGTTTCCGACCCAA	NYKLPDDFTGCVIAWNSN NLDSKVGGNYNYLYRLFR KSNLKPFERDISTEIYQAG STPCNGVKGFNCYFPLRS YSSRPR
S13	GAAGAGAATCAGCAACTGTGTTGCT GATTATTCTGTCCTATATAATTCCGC ATCATTTTCCACTTTTAAGTGTTATG GAGTGTCTCCTACTAAATTAAATGAT CTCTGCTTTACTAATGTCTATGCAGA TTCATTTGTAATTAGAGGTGATGAAG TCAGACAAATCGCTCCAGGGCAAAC TGGAAATATTGCTGATTATAATTATA AATTACCAGATGATTTTACAGGCTGC GTTATAGCTTGGAATTCTAACAATCT TGATTCTAAGGTTGGTGGTAATTATA ATTACCTGTATAGATTGTTTAGGAAG TCTAATCTCAAACCTTTTGAGAGAGA TATTTCAACTGAAATCTATCAGGCCG GTAGCACACCTTGTAATGGTGTTAAA GGTTTTAATTGTTACTTTCCTTTACG ATCATATAGTTTCCGACCCAA	VYAWNRKRISNCVADYS VLYNSASFSTFKCYGVSPT KLNDLCFTNVYADSFVIR GDEVRQIAPGQTGNIADY NYKLPDDFTGCVIAWNSN NLDSKVGGNYNYLYRLFR KSNLKPFERDISTEIYQAG STPCNGVKGFNCYFPLRS YSSRPR
R64	GTTTATGCTTGGAACAGGAAGAGAA TCAGCAACTGTGTTGCTGATTATTCT GTCCTATATAATTCCGCATCATTTTC CACTTTTAAGTGTTATGGAGTGTCTC CTACTAAATTAAATGATCTCTGCTTT ACTAATGTCTATGCAGATTCATTTGT AATTAGAGGTGATGAAGTCAGACAA ATCGCTCCAGGGCAAACTGGAAATA TTGCTGATTATAATTATAAATTACCA GATGATTTTACAGGCTGCGTTATAGC TTGGAATTCTAACAATCTTGATTCTA AGGTTGGTGGTAATTATAATTACCTG TATAGATTGTTTAGGAAGTCTAATCT CAAACCTTTTGAGAGAGATATTTCAA CTGAAATCTATCAGGCCGGTAGCAC ACCTTGTAATGGTGTTAAAGGTTTTA ATTGTTACTTTCCTTTACGATCATAT AGTTTCCGACCCAA	VYAWNRKRISNCVADYS VLYNSASFSTFKCYGVSPT KLNDLCFTNVYADSFVIR GDEVRQIAPGQTGNIADY NYKLPDDFTGCVIAWNSN NLDSKVGGNYNYLYRLFR KSNLKPFERDISTEIYQAG STPCNGVKGFNCYFPLRS YSSRPR

Table 3. SARS-CoV-2-L *isolates with their respective nucleotide and amino acid sequences.*

5.3 Sequence alignment

The amino acid sequences from the previous step were then aligned using ClustalW (GenomeNet, Kyoto University Bioinformatic Center) and analysed manually for the presence of important amino acids at certain positions that are critical for human ACE-2 binding. The sequences alignment is depicted in Figure 6 below.

Figure 6. Position of important amino acid residues believed to be involved in human ACE-2 receptor binding as highlighted in red numbers as the key binding residues (442, 472 and 479)

Based on this sequence alignment, we were able to compare the presence of critical amino acid residues critical for binding of the spike protein to human ACE-2 receptors. Based on research carried out by Hoffmann *et al.* (2020) and Zhou *et al.* (2021), they have found that specific amino acids at positions N426, Y436, Y440, L442, S456, F472, N473 and S479 are responsible for enhancing the binding of these viruses to human ACE-2 receptors with the most important positions located at L442, F472 and S479. From there, we found that amino acids of our viral isolates are similar to that those found in human SARS-CoV-2 with N426, Y436,

Y440, L442, S456, F472, N473 and S479 which indicates that SARS-CoV-2L coronaviruses isolated from bats in this study are able to bind and infect the A549 human lung cells.

5.4 Cytopathic effect in A549 human lung cells

Cytopathic effect was observed in the A549 human lung cells in all the viral samples including positive control on the day six of the post-inoculation after the third passages in the A549 cells (Figure 7). It was noted that the cells had become rounded and fused with the adjacent cells to form a syncytium. No cytopathic effect was observed in the uninoculated negative control cells (Figure 8).

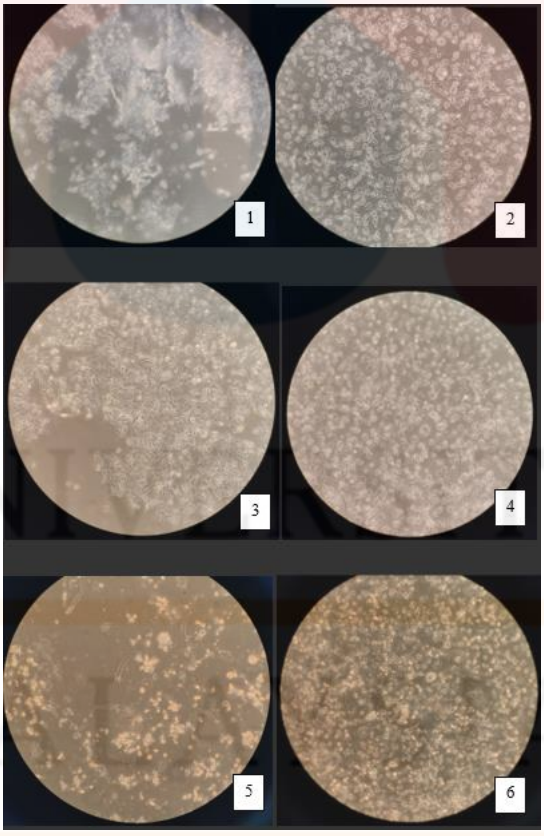

Figure 7. A549 cells with cytopathic effect on day 6 of the virus inoculation after the third passage in the cells.

Figure 7.1: A549 cells inoculated with viral sample S4.

Figure 7.2: A549 cells inoculated with viral sample S7.

Figure 7.3: A549 cells inoculated with viral sample S11.

Figure 7.4: A549 cells inoculated with viral sample S13.

Figure 7.5: A549 cells inoculated with viral sample R64.

Figure 7.6: A549 cells inoculated with positive control sample.

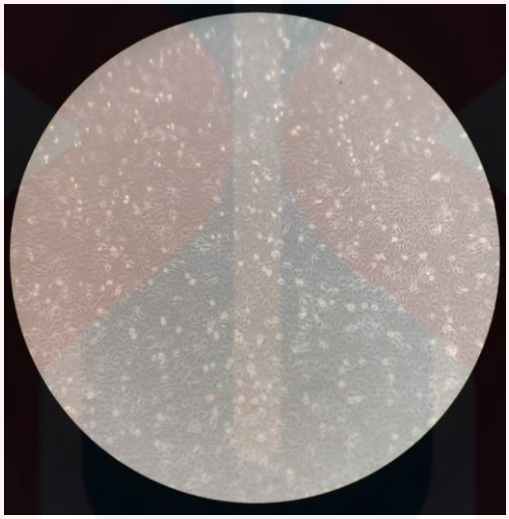

Figure 8. Normal A549 cells without cytopathic effect (negative control)

CHAPTER 6

DISCUSSION

In this experiment, five bat samples from East Coast Malaysia were tested positive for SARS-CoV-2-like coronaviruses. To determine the zoonotic potential of this virus, we amplified the RBD of the S1 subunit through RT-PCR and followed by sequencing, sequence analysis, and sequence alignment to compare the amino acid sequence of our viral samples to SARS-CoV-2 found in humans. From there, we found that amino acids critical for human ACE-2 receptor binding (N426, Y436, Y440, L442, S456, F472, N473 and S479) were present in the SARS-CoV-2L isolated from bats in this study. The amino acid residues presence was identical to those found in human SARS-CoV-2. To further support these findings, we inoculated and passaged thrice in A549 human lung cells. The isolates were abled to cause cytopathic effect on day 6 of virus inoculation. The presence of key amino acid residues coupled with the virus' ability to induce CPE in human lung cell lines revealed that these SARS-CoV-2L coronaviruses can enter the human lung cells via ACE-2 receptor and may possibly cause disease to humans.

Previous studies have determined that specific amino acid residues are key for binding of SARS-CoV-2 to human ACE-2 receptors (Hoffman *et al.*, 2020). Some bat coronaviruses, namely RaTG13, display a 96% resemblance to human SARS-CoV-2 at full genomic sequencing. Despite this similarity, such bat coronaviruses are unable to interact efficiently enough with human ACE-2 receptors to trigger infection (Zech *et al.*, 2021). The differences in the bat coronaviruses' abilities to utilize ACE-2 receptor indicate that there may have been crossover of bat coronaviruses in an intermediate host species where mutations may have occurred, allowing further crossover of the virus into humans (Gupta *et al.*, 2022). Pangolins have been suspected to be the intermediate host between bats and humans as they inhabit

similar environmental niches and sequencing of pangolin coronaviruses revealed that RBD point mutants are conserved across SARS-CoV-2-like coronaviruses isolated from bats, humans, and pangolins (Brown *et al., 2021).* On a related note, pangolin and pangolin parts are one of the most smuggled wildlife commodities in China as they are commonly used in traditional Chinese medication. A study by Lam *et al.,* 2020 isolated coronaviruses from pangolin tissue samples seized by the Guangxi Customs officers in an anti-trafficking operation. Coronaviruses were found in 5 individual pangolins which were then termed GX/P1E, GX/P2V, GX/P3B, GX/P4L, GX/P5E and GX/P5L (Lam *et al..,* 2020). Based on the genome sequences, the viruses were classified under the betacoronavirus genus. All sequences have similar genomic organisations to SARS-CoV-2, with one virus, GX/PL5 showing high binding affinity to ACE-2 receptors (Wacharapluesadee *et al.,* 2021). This is highly suggestive of pangolins as intermediate hosts for coronavirus spillover of bats into humans, however further studies should be undertaken to elucidate this claim.

In this experiment, three passages of the virus in human A549 lung cells were required before the virus was able to elicit signs of CPE in the cells. This is likely attributed to the low viral load within the archive samples thus requiring multiple passages to reach a viral load capable of inducing CPE in the cells. Based on Gilbert *et al.,* 2023, no visible CPE was noted during inoculation of virus with A549 human lung cells despite the lung cells displaying ACE-2 receptors. However, it is stipulated in this study that repeated passages of the virus in A549 human lung cells may have caused the virus to gradually increase, thus increasing the virus' ability to cause CPE in the lung cells.

In summary, we can conclude that SARS-CoV-2L isolated from bats in East Coast, Malaysia harbours important key amino acid residues at S1 RBD for binding with ACE-2 receptor in human lung cells, which allows the virus to enter the cells and thus may be capable of transmitting this virus to human hosts. Therefore, it is pertinent that extra precaution is taken

by continuous surveillance of bats in Sekayu, Terengganu and Gunung Reng, Kelantan, especially since these locations are local tourist attractions and may draw a significant number of visitors. Since Rhinolophid bats are migratory bats, studies should also be undertaken to determine the migratory paths of these bats as they may disseminate the virus on this route. The public should be made aware of the risks of being in contact with bats and to take the necessary precautions to avoid contact with bats and keep their distance from bat habitats.

CHAPTER 7

IMPORTANCE OF RESEARCH FINDINGS

These research findings showed that SARSL-CoVs isolated from bats in East Coast Malaysia have a potential to be zoonotic and become a future public health concern due to the presence of amino acid residues critical for the ACE-2 binding to human receptors. Therefore, these viruses able to enter human cells and may cause disease in the future.

CHAPTER 8

CONCLUSION & RECOMMENDATIONS

From the research findings, we found that there is presence of amino acids which are critical for human ACE-2 receptor binding in SARS-CoV-2-like coronaviruses isolated from East Coast Malaysia. Meanwhile, from virus inoculation in A549 human lung cells, it was found that the virus caused CPE in the cells on day six of inoculation. Based on these findings, we can conclude that the virus is able to bind to human ACE-2 receptors and cause cell pathology, thus confirming that the virus has zoonotic potential and may give rise to a public health threat in the future. It is recommended that the local bat populations should be kept under constant surveillance with regular screening to determine the virus status in the population. In addition, the public should be made aware of the virus' zoonotic potential and advised to maintain appropriate distance from bats and bat habitats.

APPENDIX

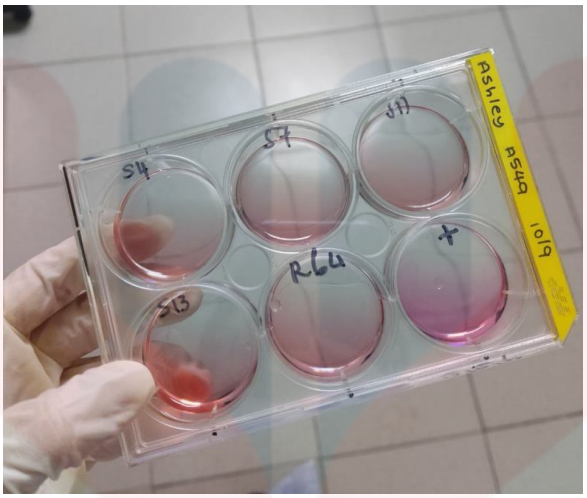

Figure 9. Seeding of virus in 6 well plate after retrieval from archives

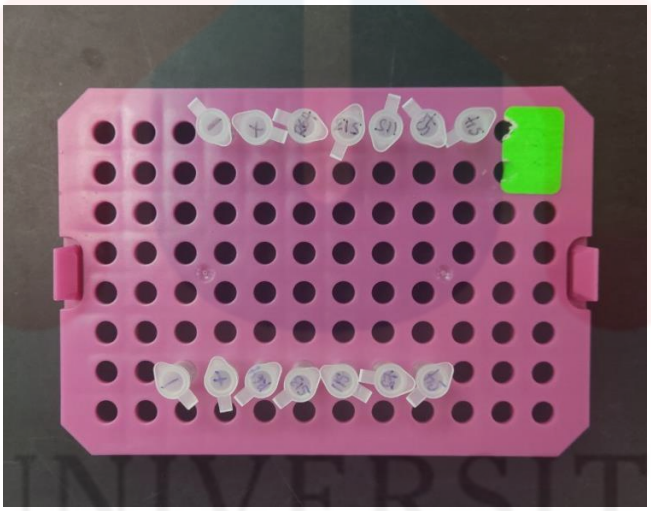

Figure 10. Preparation of samples for RT-PCR and nested PCR

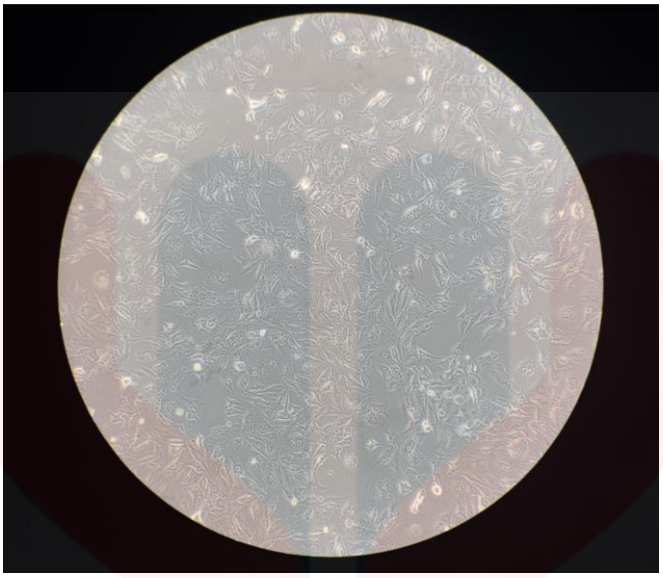

Figure 11. A549 human lung cells before inoculation with viral sample

REFERENCES

1. Brown, E. E. F., Rezaei, R., Jamieson, T. R., Dave, J., Martin, N. T., Singaravelu, R., Crupi, M. J. F., Boulton, S., Tucker, S., Duong, J., Poutou, J., Pelin, A., Yasavoli-Sharahi, H., Taha, Z., Arulanandam, R., Surendran, A., Ghahremani, M., Austin, B., Matar, C., Diallo, J. S., … Azad, T. (2021). Characterization of Critical Determinants of ACE2-SARS CoV-2 RBD Interaction. *International journal of molecular sciences*, *22*(5), 2268. Sourced from: https://doi.org/10.3390/ijms22052268

2. de Groot, R. J., Baker, S. C., Baric, R. S., Brown, C. S., Drosten, C., Enjuanes, L., Fouchier, R. A., Galiano, M., Gorbalenya, A. E., Memish, Z. A., Perlman, S., Poon, L. L., Snijder, E. J., Stephens, G. M., Woo, P. C., Zaki, A. M., Zambon, M., & Ziebuhr, J. (2013). Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. Journal of virology, 87(14), 7790–7792. Sourced from: https://doi.org/10.1128/JVI.01244-13

3. Grudlewska-Buda, K., Wiktorczyk-Kapischke, N., Wałecka-Zacharska, E., Kwiecińska-Piróg, J., Buszko, K., Leis, K., Juszczuk, K., Gospodarek-Komkowska, E., & Skowron, K. (2021). SARS-CoV-2-Morphology, Transmission and Diagnosis during Pandemic, Review with Element of Meta-Analysis. Journal of clinical medicine, 10(9), 1962. Sourced from: https://doi.org/10.3390/jcm10091962

4. Gupta, S. K., Minocha, R., Thapa, P. J., Srivastava, M., & Dandekar, T. (2022). Role of the Pangolin in Origin of SARS-CoV-2: An Evolutionary Perspective. International journal of molecular sciences, 23(16), 9115. Sourced from: https://doi.org/10.3390/ijms23169115

5. Han HJ, Wen HL, Zhou CM, Chen FF, Luo LM, Liu JW, Yu XJ. Bats as reservoirs of severe emerging infectious diseases. Virus Res. 2015 Jul 2;205:1-6. doi:

10.1016/j.virusres.2015.05.006. Epub 2015 May 18. PMID: 25997928; PMCID: PMC7132474.

6. Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., ... & Pöhlmann, S. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *cell*, *181*(2), 271-280.
7. Hu, B., Ge, X., Wang, L. F., & Shi, Z. (2015). Bat origin of human coronaviruses. Virology journal, 12, 221. Sourced from: https://doi.org/10.1186/s12985-015-0422-1.
8. Jackson, C.B., Farzan, M., Chen, B. et al. Mechanisms of SARS-CoV-2 entry into cells. Nat Rev Mol Cell Biol 23, 3–20 (2022). Sourced from: https://doi.org/10.1038/s41580-021-00418-x
9. Lam, T.TY., Jia, N., Zhang, YW. *et al.* Identifying SARS-CoV-2-related coronaviruses in Malayan pangolins. *Nature* 583, 282–285 (2020). Sourced from: https://doi.org/10.1038/s41586-020-2169-0
10. Letko, M., Seifert, S.N., Olival, K.J. et al. Bat-borne virus diversity, spillover and emergence. Nat Rev Microbiol 18, 461–471 (2020). Sourced from: https://doi.org/10.1038/s41579-020-0394-z
11. Li, W. (2005). *Bats Are Natural Reservoirs of SARS-Like Coronaviruses. Science, 310(5748), 676–679.* doi:10.1126/science.1118391
12. Li F, Li W, Farzan M, Harrison SC. Structure of SARS coronavirus spikereceptor-binding domain complexed with receptor. Science. 2005;309(5742): 1864–8. doi:10.1126/science.1116480.
13. Liu, Y., Kuo, R., & Shih, S. (2020). COVID-19: The first documented coronavirus pandemic in history. Biomedical Journal, 43(4), 328-333. Sourced from: https://doi.org/10.1016/j.bj.2020.04.007

14. Looi LM, Chua KB. Lessons from the Nipah virus outbreak in Malaysia. Malays J Pathol. 2007 Dec;29(2):63-7. PMID: 19108397.
15. Mannar, D., Saville, J. W., Zhu, X., Srivastava, S. S., Berezuk, A. M., Zhou, S., Tuttle, K. S., Kim, A., Li, W., Dimitrov, D. S., & Subramaniam, S. (2021). Structural analysis of receptor binding domain mutations in SARS-CoV-2 variants of concern that modulate ACE2 and antibody binding. Cell reports, 37(12), 110156. Sourced from: https://doi.org/10.1016/j.celrep.2021.110156
16. Priyadarshi, R., Purohit, S. D., Roy, S., Ghosh, T., Rhim, J.-W., & Han, S. S. (2022). Antiviral Biodegradable Food Packaging and Edible Coating Materials in the COVID-19 Era: A Mini-Review. Coatings, 12(5), 577. Sourced from: https://doi.org/10.3390/coatings12050577
17. Reduan, F. H., Tan, L. P., & Jusoh, M. (2023). RESEARCH ARTICLE Presence of SARS-CoV-2-like coronaviruses in bats from east coast Malaysia. *Tropical biomedicine*, *40*(3), 273-280.
18. Ruiz-Aravena, M., McKee, C., Gamble, A. et al. Ecology, evolution and spillover of coronaviruses from bats. Nat Rev Microbiol 20, 299–314 (2022). Sourced from: https://doi.org/10.1038/s41579-021-00652-2
19. Shang, J., Wan, Y., Luo, C., Ye, G., Geng, Q., Auerbach, A., & Li, F. (2020). Cell entry mechanisms of SARS-CoV-2. Proceedings of the National Academy of Sciences of the United States of America, 117(21), 11727–11734. Sourced from: https://doi.org/10.1073/pnas.2003138117
20. Temmam, S., Vongphayloth, K., Baquero, E. et al. Bat coronaviruses related to SARS-CoV-2 and infectious for human cells. Nature 604, 330–336 (2022). Sourced from: https://doi.org/10.1038/s41586

21. V'kovski, P., Kratzel, A., Steiner, S. et al. Coronavirus biology and replication: implications for SARS-CoV-2. Nat Rev Microbiol 19, 155–170 (2021). Sourced from: https://doi.org/10.1038/s41579-020-00468-6

22. Wacharapluesadee, S., Tan, C.W., Maneeorn, P. *et al.* Evidence for SARS-CoV-2 related coronaviruses circulating in bats and pangolins in Southeast Asia. *Nat Commun* **12**, 972 (2021). https://doi.org/10.1038/s41467-021-21240-1

23. Wan, Y., Shang, J., Graham, R., Baric, R. S., & Li, F. (2020). Receptor Recognition by the Novel Coronavirus from Wuhan: an Analysis Based on Decade-Long Structural Studies of SARS Coronavirus. Journal of virology, 94(7), e00127-20. Sourced from: https://doi.org/10.1128/JVI.00127-20

24. Wang, C. C., Prather, K. A., Sznitman, J., Jimenez, J. L., Lakdawala, S. S., Tufekci, Z., & Marr, L. C. (2021). Airborne transmission of respiratory viruses. Science (New York, N.Y.), 373(6558), eabd9149. Sourced from: https://doi.org/10.1126/science.abd9149

25. Zech, F., Schniertshauer, D., Jung, C., Herrmann, A., Xie, Q., Nchioua, R., ... & Kirchhoff, F. (2021). Spike mutation T403R allows bat coronavirus RaTG13 to use human ACE2. BioRxiv, 2021-05.

26. Zhou, H., Ji, J., Chen, X., Bi, Y., Li, J., Wang, Q., Hu, T., Song, H., Zhao, R., Chen, Y., Cui, M., Zhang, Y., Hughes, A. C., Holmes, E. C., & Shi, W. (2021). Identification of novel bat coronaviruses sheds light on the evolutionary origins of SARS-CoV-2 and related viruses. *Cell*, *184*(17), 4380–4391.e14. Sourced from: https://doi.org/10.1016/j.cell.2021.06.008

27. Zhou, P., Yang, XL., Wang, XG. et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 579, 270–273 (2020). Sourced from: https://doi.org/10.1038/s41586-020-2012-7

28. Zhou, Z. M., Zhou, H. Z., Lin, X. D., Su, Z. C., Zhao, L. S., & Chen, X. (2020). Outbreak of COVID-19 in a family, Wenzhou, China. Epidemiology and infection, 148, e103. Sourced from: https://doi.org/10.1017/S0950268820001089

29. Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., Niu, P., Zhan, F., Ma, X., Wang, D., Xu, W., Wu, G., Gao, G. F., & Tan, W. (2020). A novel coronavirus from patients with pneumonia in China, 2019. New England Journal of Medicine, 382(8), 727-733 https://doi.org/10.1056/nejmoa2001017