
UNIVERSITI PUTRA MALAYSIA

# DETECTION OF *CORONAVIRIDAE* VIRUS RNA IN *RATTUS* SPP. IN KLANG VALLEY

NURUL AFIQAH BINTI HUSAIN

# DETECTION OF *CORONAVIRIDAE* VIRUS RNA

# IN *RATTUS* SPP. IN KLANG VALLEY

**NURUL AFIQAH BINTI HUSAIN**

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
Universiti Putra Malaysia
Serdang, Selangor Darul Ehsan.

2022

**CERTIFICATION**

It is hereby certified that we have read this project paper entitled “Detection of *Coronaviridae* virus RNA in *Rattus* spp. In Klang Valley”, by Nurul Afiqah binti Husain and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Final Year Project.

_____

**DR. NUR FAZILA SAULOL HAMID**
**DVM (UPM), PHD (UNIVERSITY OF LIVERPOOL),**
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

_____

DR. NOR YASMIN ABD RAHAMAN
DVM (UPM), PHD (UPM)
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

PROF. DR. SITI SURI ARSHAD
DVM (UPM), PHD (UNIVERSITY OF LONDON)
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

## DEDICATION

My humble effort I dedicate to my beloved parents, *Mr. Husain Mohamad Nor and Mrs. Missiah Ali,* and my siblings, cats and family for their love, motivation, and endless support throughout the many years of my education until this point.

To my long list of friends, those in the same course and the rest, who have provided me with overwhelming support, love, and encouragement to continue motivating me to complete this project paper.

## ACKNOWLEDGEMENT

A big thank you to my great lecturers, Dr. Nur Fazila Saulol Hamid, Dr. Nor Yasmin Abd Rahaman and Prof. Dr. Siti Suri Arshad for their guidance, encouragement, patience, time, and invaluable help in preparing this thesis.

I would like to express my gratitude towards my family and my furry friends, Mamao and Mimik for their endless untiring love and support which helped and motivated me in finishing this thesis.

I am most grateful to my friends, especially Raja, Anis, and Casyndra. Thank you for their emotional support and their helpful suggestions for improvements from my original ideas.

I would also like to thank my FYP teammates, Tasya, Imah and Tisha. The completion of this project would not have been possible without the help and support from them.

I would also like to express my gratitude to the postgraduate students, Natasha and Faiz who had helped throughout the final year project journey.

## CONTENTS

**LIST OF TABLES** **Page**



**LIST OF FIGURES**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| % | percentage |
| °C | degree celsius |
| x g | gravitational force |
| µL | microlitre |
| kb | kilobase |
| mA | milliampere |
| mL | millimetre |
| V | voltage |
| bp | base pair |
| BCoV | *Betacoronavirus* |
| COVID-19 | Coronavirus Disease 2019 |
| DNA | Deoxyribonucleic acid |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| RdRp | RNA dependent polymerase |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| RT-qPCR | Reverse transcription quantitative real-time PCR |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| sp. | particular species |
| spp. | several species |
| TAE | Tris-acetate-EDTA |

## ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir.

PENGESANAN RNA VIRUS *CORONAVIRIDAE* DALAM *RATTUS* SPP. DI LEMBAH KLANG

Oleh

NURUL AFIQAH BINTI HUSAIN

2022

Penyelia: Dr. Nur Fazila Saulol Hamid

Penyelia bersama: Dr. Nor Yasmin Abd Rahaman dan Prof. Dr. Siti Suri Arshad

Kepentingan *Coronaviridae* di dalam haiwan sebagai potensi ancaman dan penyakit zoonosis yang membawa maut telah ditekankan sejak wabak COVID-19. Tikus mempunyai populasi yang tinggi dan tinggal berdekatan dengan manusia, meningkatkan risiko penularan patogen. Coronavirus di dalam *Rattus spp.* menjangkiti saluran pernafasan, mata, kelenjar lakrimal dan air liur. Penyakit ini adalah penyakit berjangkit dan boleh menjangkiti kepada tikus yang terdedah melalui sentuhan langsung, fomit atau aerosol. Walaubagaimanapun, saringan RNA *Coronaviridae* di dalam tikus masih belum dijalankan di Malaysia. Oleh itu, kajian ini

bertujuan untuk mengesan kehadiran RNA *Coronaviridae* dalam tikus di Lembah Klang. Sebanyak 35 tikus ditangkap daripada pasar-pasar di kawasan Lembah Klang telah diambil sampel untuk serum darah dan swab orofarinks. RNA telah diekstrak menggunakan reagen TRIzol™, diikuti oleh ujian semi-nested reaksi rantai polimerase transkriptase membalik (RT-PCR) menyasarkan gen RNA-Dependent RNA polymerase (RdRp) dan terakhir elektroforesis gel. Amplicon tepat-tepat 440bp dijangka daripada pusingan pertama dan 434bp daripada pusingan kedua. Di dalam kajian ini, tiada sampel swab positif untuk RNA virus *Coronaviridae*. Walaubagaimanapun, 4 daripada 35 sampel serum disyaki positif kerana saiz jalur tidak jauh beza dengan kawalan positif. Separa "gene sequencing" menunjukkan bahawa keempat-empat sampel negatif untuk RNA virus *Coronaviridae*. Ia mungkin disebabkan oleh keadaan tidak viraemik, saiz sampel yang rendah dan jenis sampel yang terhad. Walaupun RNA *Coronaviridae* tidak dapat dikesan dalam kajian ini, potensi risiko untuk zoonosis dan keupayaan virus untuk mengatasi halangan spesies dan menyesuaikan diri dengan perumah baharu tidak boleh diabaikan.

**Kata kunci: tikus; *Coronaviridae;* "semi-nested" RT-PCR; zoonosis; Lembah Klang**

## ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Final Year Project.

**DETECTION OF *CORONAVIRIDAE* VIRUS RNA IN *RATTUS* SPP. IN KLANG VALLEY**

**By**

**NURUL AFIQAH BINTI HUSAIN**

**2022**

**Supervisor: Dr. Nur Fazila Saulol Hamid**

**Co-supervisors: Dr. Nor Yasmin Abd Rahaman and Prof. Dr. Siti Suri Arshad**

The importance of *Coronaviridae* in animals as a potential threat and deadly zoonotic disease has been highlighted since the pandemic of COVID-19. Rats are highly populated and live in close proximity to humans, increasing the risk of pathogen transmission. Coronavirus in *Rattus* spp. infects the respiratory tract, eyes, lacrimal and salivary glands that could be transmitted through direct contact, fomites or aerosol. However, screening of *Coronaviridae* RNA in rats has not been conducted in Malaysia. Therefore, this study aims to conduct a preliminary screening to detect the presence of *Coronaviridae* RNA in rats in Klang Valley. A total of 35 rats collected

from selected markets in Klang Valley were sampled for blood serum and oropharyngeal swabs. The RNA was extracted using a TRIzol™ reagent, followed by a semi-nested reverse-transcription polymerase chain reaction (RT-PCR) assay targeting the RNA-dependent RNA polymerase (RdRp) gene and lastly gel electrophoresis. Amplicon at approximately 440bp was expected from the first round and 434bp from the second round. The results showed none of the swab samples was positive for *Coronaviridae* RNA, however, 4 out of 35 serum samples suspected to be positive were subjected to partial gene sequencing also revealed negative results. It is possibly due to the non-viraemic state, low sample size, and limited sample types. Although *Coronaviridae* RNA is undetected in this study, the potential of zoonotic risk and the ability of the virus to overcome the species barriers and adapt to new hosts shall not be ignored.

**Keywords:** rats; *Coronaviridae*; semi-nested RT-PCR; zoonotic; Klang Valley

# 1.0 INTRODUCTION

## 1.1 Background

Since 2019, many countries have been affected by the pandemic wave of COVID-19 which started in Wuhan City, Hubei province of China. Studies have shown that *Coronaviridae* has repeatedly crossed species barriers, broadening its host range as it constantly evolves to cause human infections (Nova, 2021). It leads to a concern about whether rodents could transmit the *Coronaviridae* to humans as they can harbour different types of pathogens.

*Murine coronavirus* is the type of species that can be found in rats under the genus *Betacoronavirus* of the family *Coronaviridae*. There are several distinct serotypes under this species which are murine hepatitis virus and rat coronaviruses. The transmission of this virus to the susceptible rats is through direct contact with infected rats or indirectly through aerosol and fomites (Otto et al., 2015). Coronavirus in *Rattus* spp. infects the respiratory tract, eyes, lacrimal and salivary glands. All ages of rats are susceptible to disease as they are very contagious but it is most severe in young rats (MacLachlan et al., 2016).

The population of rodents in urban areas that live in close vicinity to the public are increasing nowadays. This issue raises a concern about the risk to public health towards zoonotic transmission as rodents are well-known as zoonotic reservoirs (Meerburg et al., 2009). Since they are synanthropic, exposure to food supplies may attract them and zoonotic transmission may happen via direct or indirect contact with rodent faeces or saliva (Camp et al., 2022). A study reported that people in Vietnam and Cambodia consume rats weekly as food due to their delicious taste, cheapness and perceived to have health benefits, increasing more zoonotic risk. The origin of

*Coronaviridae*-related diseases that affect humans mostly originated from animals, specifically wild animals (Huong et al., 2020). Coronavirus can cause respiratory, hepatic, enteric and neurological disease not only in animal species but in humans too (Wang et al., 2020). Therefore, early detection of *Coronaviridae* in rats is very important to prevent a new disease outbreak.

## 1.2 Justification

Rodents are a major zoonotic source of human infectious disease as they harbour different types of pathogens. Since the population of rodents living in close proximity to humans increases, the zoonotic risk is higher when directly or indirectly exposed to rodent carcasses, faeces, urine and parasites. They can be a reservoir or an intermediate host for *Coronaviridae* virus and possible to transmit it to humans. The prevalence of *Coronaviridae* in rats has not yet been reported in Malaysia. Therefore, this preliminary study is extremely essential to determine the presence of *Coronaviridae* in the rodent population in Klang Valley.

## 1.3 Objective

The study aimed to detect presence of *Coronaviridae* RNA in *Rattus* spp. in Klang Valley using semi-nested RT-PCR method.

## 1.4 Hypothesis

$H_o$: There is no detection of *Coronaviridae* RNA from *Rattus* spp. in Klang Valley.

$H_A$: There is no detection of *Coronaviridae* RNA from *Rattus* spp. in Klang Valley.

## 2.0 LITERATURE REVIEW

### 2.1 Properties of *Coronaviridae*

*Coronaviridae* is a group of single-stranded, positive-strand RNA genome viruses that can infect a diverse range of animals and also humans. They are spherical or pleomorphic virions with a diameter of 125 nm approximately. The length of the genome varies from 26 to 32 kilobases (kb) which is the largest genome among RNA viruses. They are non-segmented, enveloped viruses with club-shaped spikes on their surface giving the appearance of a crown which reflects their name as the corona. They have 4 or 5 structural proteins and 16 non-structural proteins. The four major structural proteins in the genome are nucleocapsid (N), envelope (E), membrane (M) and spike (S) protein (Figure 1). The largest structural protein which is the S protein mediates the attachment of the virus to the host cell surface receptors (Malik, 2020).

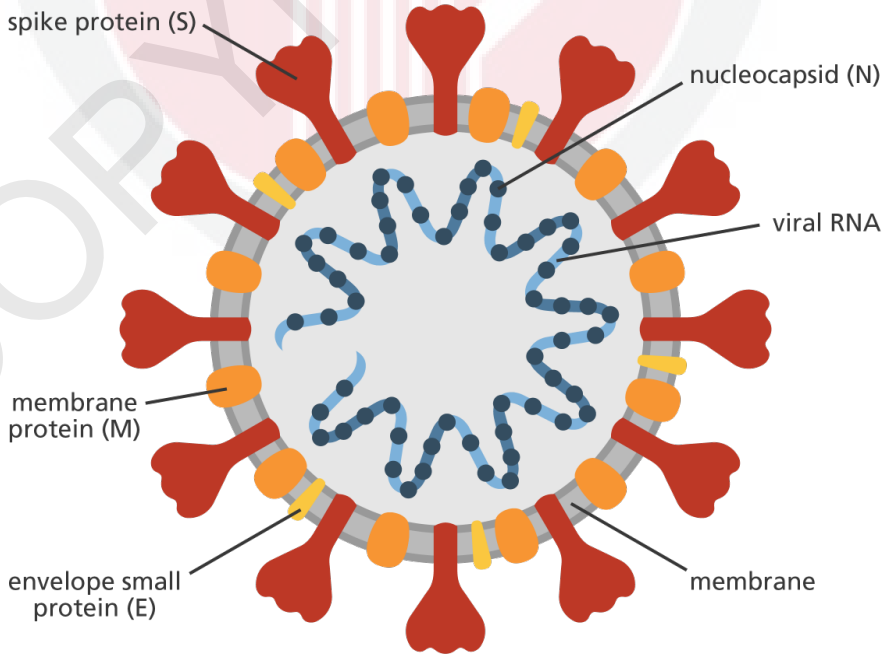



Figure 1. Virion structure diagram (subfamily *Coronavirinae*). Adapted from *Genome Research Limited.* https://www.yourgenome.org/facts/what-is-covid-19/

Family *Coronaviridae* under the order Nidovirales is classified into two subfamilies which are *Orthocornavirinae* and *Torovirinae.* The subfamily *Orthocoronavirinae* can be further divided into four genera which are *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. *Torovirinae* only have two genera under its subfamily which are *Torovirus* and *Bafinivirus* (Payne, 2017). Most of the viruses in the genus *Alphacoronavirus* and *Betacoronavirus* only infect mammals, while *Gammacoronavirus* infects avian species and *Deltacoronavirus* can be found in both mammals and avian species (de Groot et al., 2011). Feline coronavirus (FCoV), canine coronavirus (CCoV), murine hepatitis virus (MHV), infectious bronchitis virus (IBV) and porcine epidemic diarrhoea virus (PEDV) are all examples of common viruses under this family. As this study focused on rats, all rat coronavirus belongs to *Betacoronavirus* under subgenus *Embecovirus* (Figure 2).

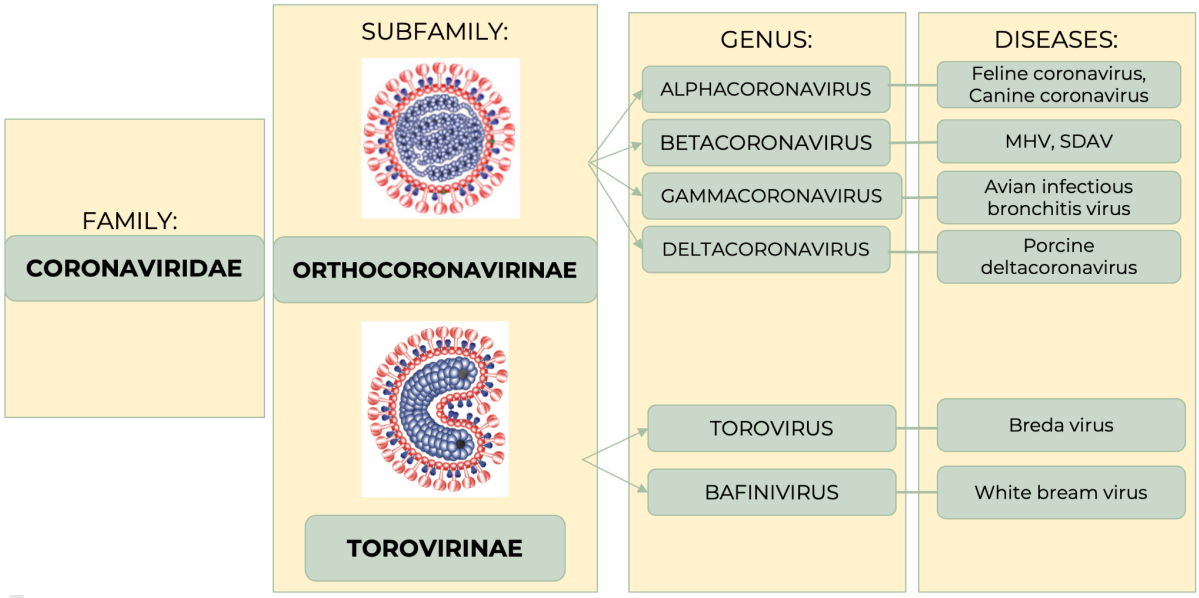



Figure 2. Classification of Family *Coronaviridae*.

## 2.2 *Coronaviridae* in rats

A novel member of the coronavirus family was first reported in the 1960s, when laboratory rats showed clinical signs of severe sialadenitis, dacryoadenitis and lower respiratory tract infection (Jonas et al., 1969). In 1964, Hartley's team discovered the agent in the serum of rats possibly related to the mouse hepatitis virus (MHV) antigenically (Hartley et al., 1964). This finding was then further confirmed by Jonas et al. (1969) who studied a pathogenic agent and identified virus-like particles using an electron microscope in the infected salivary glands of rats. They managed to isolate Parker's rat coronavirus from the lungs of asymptomatic rats in their continuation of studies (Parker et al., 1970). Sialodacryoadenitis (SDA) was the second novel strain that was discovered similarly antigenically related to MHV and Parker rat coronavirus (Bhatt et al., 1972). In a deeper dive into this subject, there were several other strains managed to be discovered in other countries.

Rat coronavirus can be asymptomatic or symptomatic in rats. It can be transmitted to susceptible rats through direct contact with infected individuals or through indirect contact by aerosol or fomites. This was proven through an experimental study done by Gaertner et al. (1993) where the virus persisted when dried on a solid surface for up to two days. However, there is no evidence of intrauterine transmission. The tissue tropisms for rat coronavirus are salivary glands, lacrimal glands, Harderian glands and respiratory epithelium. The symptoms can last as long as two weeks and complications occur if the rats are infected with the herpes virus which leads to keratitis and megaglobus. Other clinical signs consist of edema of submaxillary salivary glands, nasal and ocular discharge with porphyrin stains, lacrimation, photophobia, corneal opacities, corneal ulcers and cervical swelling due to inflammation. Additional signs may include temporary anorexia and weight loss and

reproductive problems such as disruption of estrus and reduced fertility (Bartak et al., 2021).

2.3 Surveillance of *Coronaviridae* in rats

Surveillance of *Coronaviridae* or specifically coronaviruses in rats has been conducted for the past few years in a few countries around the world (Figure 3). Most studies were related to the pandemic of COVID-19 due to the emerging outbreak of coronaviruses in the past two decades to discover whether rats could become the potential reservoirs for coronavirus.

In Vietnam, surveillance of coronavirus has been done in field rats such as *Rattus* sp., *Bandicota* sp. and bamboo rats that are used for consumption as meat. The samples that were used in this study were faeces, urine and oral swabs and conducted through nested-PCR assay targeting the RdRp gene. As for the results, 239 out of 702 (34.0%) rats were found positive for coronavirus, specifically murine coronavirus and Lonquan Aa coronavirus (Huong et al., 2020).

Previous studies done in Belgium and Canada were conducted to detect SARS-CoV-2 in rats. These two studies used different types of samples where oral swabs, fecal and tissue samples were used in Belgium. Meanwhile, in Canada, nasal turbinates, intestinal and thoracic cavity fluid were used to detect SARS-CoV-2. Different molecular tests were also used such as RT-PCR and RT-qPCR in Canada and Belgium respectively. However, both of these studies found none of the rodents was positive for *Coronaviridae* (Colombo et al., 2021; Robinson et al., 2022).

Semi-nested RT-PCR was also another molecular test that was used to detect *Coronaviridae* which was conducted in studies done in Laos and Canary Islands. Oral and rectal swabs and fecal samples were used to detect *Coronaviridae* in Laos and Canary Islands respectively. In Laos, 12 out of 851 rats (1.41%) were detected positive for coronavirus. Meanwhile, the rats that they discovered in the Canary Islands were positive for murine coronavirus (McIver et al., 2020; Monastiri et al., 2021).

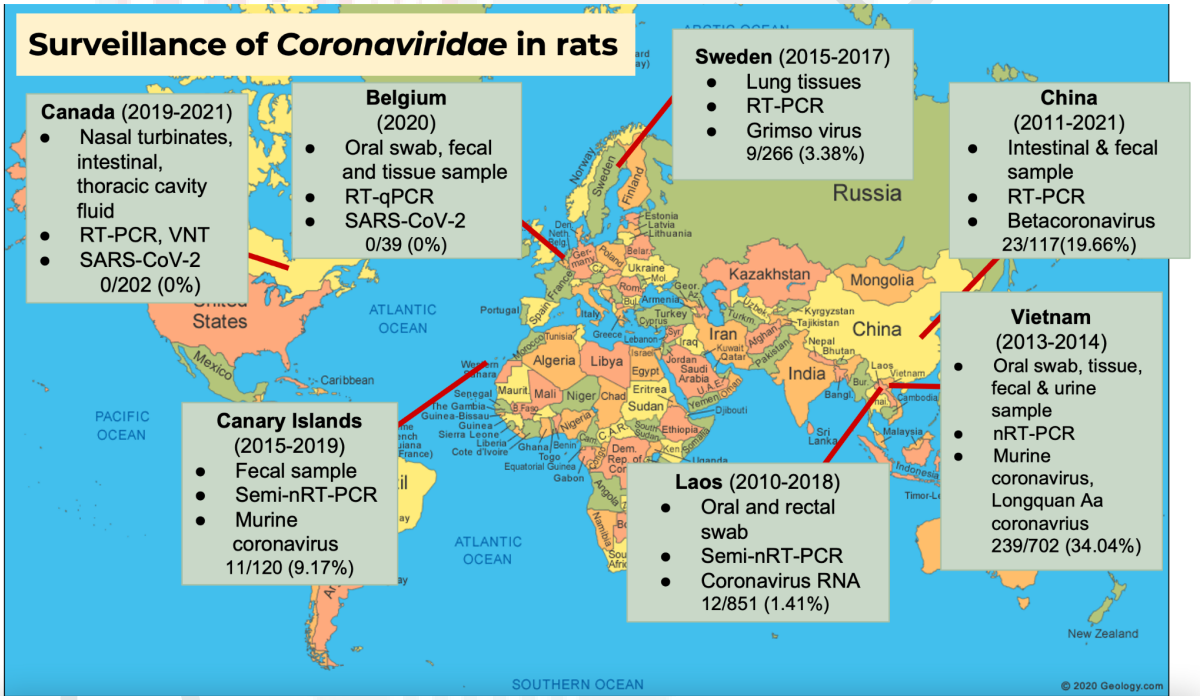



Figure 3. *Coronaviridae* surveillance in rats around the world.

2.4 Discovery of novel *Coronaviridae* in rats

The discovery of a new *Betacoronavirus* from the bank voles (*Myodes glareolus*), which is mainly found in Grimsö and named the virus as Grimso virus, has been reported in Sweden. The virus is ubiquitous in bank voles and has been tested repeatedly for three years, with an overall prevalence of 3.4% in bank voles. A total of 266 bank voles were collected for their lung samples and analysed by RT-PCR

where the specific primers target the spike gene protein. Only 9 out of 266 bank voles were positive for the Grimso virus (Wasberg et al., 2022).

Discovery of novel *Coronaviridae* of rats in China is illustrated in Figure 4. Based on previous studies, a new lineage of A *Betacoronavirus* (A BCoV), China *Rattus* Coronavirus (ChRCoV) HKU24 had been discovered and isolated in southern China from Norway rats in 2014. Bats and birds are usually the most common ancestors of coronaviruses. However, in this study, neither the birds nor the bats had found lineage A BCoV in them except for rodents. Therefore, ChRCoV HKU24 may represent the murine ancestor for *Betacoronavirus 1* and rodents are most likely an important reservoir for ancestors of lineage A BCoVs. The spreading of ChCoV HKU24 infection from rodents to other mammals via interspecies transmission is also possible (Lau et al., 2015).

Another study also reported that three novel *Coronaviridae* had been discovered during a survey for coronavirus in rats in China. They are Lucheng Rn rat coronavirus (LRNV), Longquan Aa mouse coronavirus (LAMV) and Longquan R1 rat coronavirus (LRLV). LAMV and LRLV fell into lineage of A BCoV while LRNV found in lineage of *Alphacoronavirus* harboured by *Rattus norvegicus*. The coronavirus were detected from five species of rats which were *Apodemus agrarius*, *Rattus norvegicus*, *Rattus lossea*, *Rattus tanezumi* and *Niviventer confucianus* by using RT-PCR targeting RdRp gene and further confirmed through genetic analyses. This shows increasing numbers of rodent-associated *Coronaviridae* have been identified in many countries and in a range of rodent species (Wang et al., 2015). Therefore, there is a possibility that the rodents are carrying unidentified CoVs circulating among animals.


Figure 4. Discovery of novel *Coronaviridae* in rats in China.

## 3.0 MATERIALS AND METHODS

### 3.1 Ethical Approval

This study was approved by the UPM Institutional Animal Care and Usage Committee (IACUC), with an approval code of UPM/IACUC/AUP-R021/2021.

### 3.2 Sample collection

From April to June 2022, 35 rats were trapped from three selected market areas in Klang Valley which include the Pasar Pudu, Pasar Borong Selangor and Pasar Sri Serdang. During sampling, complete personal protective equipment was worn. The baits that were used to trap the rats were coconut flesh with bread and salted fish. The traps were prepared at the market in the evening and then collected the next morning. The trapped rats (n = 35) were brought to the Post Mortem Laboratory, Faculty of Veterinary Medicine (FPV), UPM, for further sample collection.

The rats were sampled based on those previously described by Mohd-Qawiem et al., 2022. The blood samples were processed to obtain the serum in the Laboratory of Veterinary Virology, FPV. The serum samples were incubated for 24 hours at room temperature and then centrifuged at 1000 x g for 10 minutes at room temperature (Eppendorf, Germany). The serum was transferred to 1.5mL tubes (Eppendorf, Germany) before storage in the -80$^{o}$C freezer (SANYO Ultra Low, Japan) until further use. The sterile cotton swab tube (BD Vacutainer®, New Jersey, USA) was used to collect the oropharyngeal swabs before it was transferred into 2 ml of PBS as transport media. The samples were stored in a -80$^{o}$C freezer until they were used for serological analysis. Morphometric identification was done where the rats were

identified based on the measurement of their body weight, body height, tail, hind foot, ear and number of nipples. The species, age, and sex of the rats are recorded in Table 1.

Table 1 Identification of rodents

| Rodent species | Sex | | Age | |
|---|---|---|---|---|
| | Male | Female | Adult | Juvenile |
| *Rattus rattus* | 11 | 9 | 12 | 8 |
| *Rattus norvegicus* | 3 | 7 | 10 | 0 |
| *Suncus murinus* | 3 | 2 | 1 | 4 |
| Total | 17 | 18 | 23 | 12 |

3.3 RNA Extraction

Viral RNA was extracted from serum samples and oropharyngeal swabs using TRIsure™ (Bioline, United Kingdom). The RNA was eluted in 20 µl sterile dd$H^2O$ and was used as the template for semi-nested RT-PCR. RNA purity and concentration were determined using the BioPhotometer (Eppendorf, Germany) at the absorbance value of 260/280 nm. The average value of 1.8 to 2.1 indicates a good purity of nucleic acid.

### 3.4 Semi-nested Reverse Transcription Polymerase Chain Reaction (RT-PCR) for detection of *Coronaviridae* targeting RdRp gene

Detection of *Coronaviridae* was done through a semi-nested reverse transcription polymerase chain reaction which consists of RT-PCR round 1 and PCR round 2. The primers were used to target the conserved region of the RNA-dependent RNA polymerase (RdRp) gene which is a highly conserved region across all CoV genera and encodes essential enzymes for viral RNA replication. A synthetic plasmid was used as the positive control with the addition of a 20 µL master mix. RT-PCR round 1 or known as one-step RT-PCR was performed using MyTaq™ One-Step RT-PCR (Bioline, United Kingdom) in a 25µL reaction. The expected PCR product for the RT-PCR round 1 is 440 bp.

PCR round 2 was performed using MyTaq™ Red Mix (Bioline, United Kingdom) by preparing a master mix reaction of 12.5µL MyTaq™ Red Mix, 5.5µL dd$H_2O$ and 1 µL each of the forward and reverse primers. Table 2 lists the primer sequences that target the RdRp gene to detect *Coronaviridae*. The mixtures were then amplified in the PCR machine (Eppendorf, Germany) based on PCR protocols for *Coronaviridae* by Watanabe et al. (2010) where the initialising step at 95°C for 1 minute, denaturation step at 95°C for 15 seconds, followed by annealing with 34 PCR cycles of 50°C for 15 seconds, extension at 72°C for 10 seconds and lastly the final extension step at 72°C for 5 minutes. The expected PCR product for round 2 is 434 bp. Standard precautions were taken to avoid PCR contamination and no false positive was observed in negative controls.

Table 2 Forward and reverse primers used in RT-PCR and PCR analysis for detection of *Coronaviridae* RNA.

| PCR Reaction | Primer | Primer sequence (5'-3') | Product size (bp) | Reference |
|---|---|---|---|---|
| RT-PCR Round 1 | Forward | GGTTGGGAYTAYCCHAARTGTGA | 440 | Watanabe et al. (2010) |
| | Reverse | CCATCATCASWYRAATCATCATA | | |
| PCR Round 2 | Forward | GAYTAYCCHAARTGTGAUMGWGC | 434 | |
| | Reverse | CCATCATCASWYRAATCATCATA | | |

3.5 Gel Electrophoresis

Gel electrophoresis was performed using 2.0% gel prepared by mixing agarose gel powder in TAE (Tris-acetate-EDTA) buffer solution and Red Safe™ (South Korea) to separate the PCR products. The gel electrophoresis was set at 90V and 350mA for 35 minutes (Bio-Rad, California). The DNA fragments were then observed under the UV light transilluminator (Syngene, United Kingdom).

3.6 Partial Gene Sequencing

Any suspected positive results were subjected to partial gene sequencing (1st BASE, Selangor, Malaysia). Sequences of the PCR products obtained were screened against the GenBank nucleotide database using the MegaBlast search function of the National Centre for Biotechnology Information (NCBI) to compare the query sequences and obtain identical nucleotide sequences.

## 4.0 RESULTS

### 4.1 RT-PCR assay

Based on the result of RT-PCR analysis of the oropharyngeal swab sample, all 35 rats were negative for *Coronaviridae* as no band was observed at the 434-base pair (bp) when compared to the positive control (Figure 1). However, the result of RT-PCR analysis for the serum sample, 4 out of 35 rats were suspected to be positive as there was the presence of a faint band near the positive control observed at the 434 bp (Figure 2). The multiple bands observed in Figure 2 indicate the presence of other general families of viruses, which were included in this study as universal controls and were capable of detecting a wide range of DNA templates. Another study also stated that the presence of multiple bands in PCR can be due to nonspecific primer annealing or DNA contamination. Having too many PCR cycles can also lead to numerous bands because there is a higher possibility of getting the error with each cycle (Bovo et al., 1999).

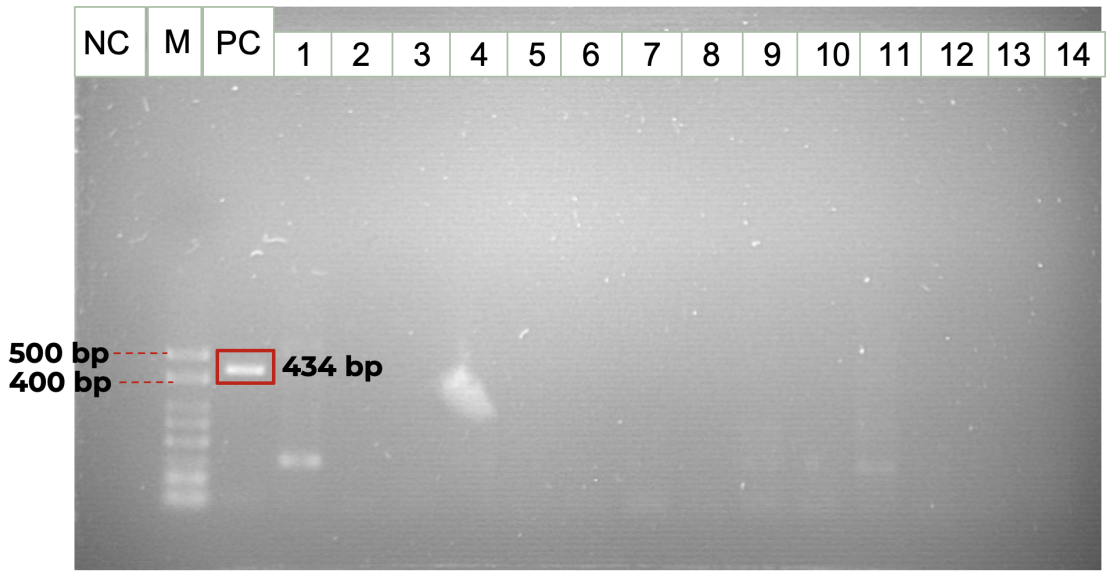



Figure 5. Gel electrophoresis of oropharyngeal swab analysis to detect *Coronaviridae* RNA targeting RdRp gene. No bands are observed for all samples. *Note.* NC = Negative control; M = Ladder; PC = Positive control

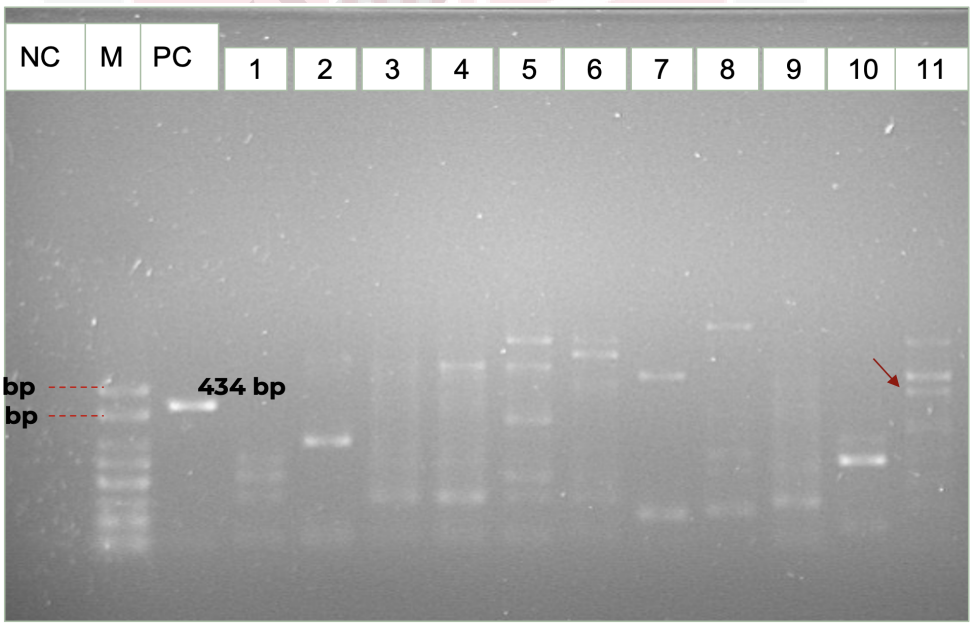



Figure 6. Gel electrophoresis of sera analysis to detect *Coronaviridae* RNA targeting RdRp gene. Sample 11 (red arrow) showed the presence of a faint band near 434 bp. *Note.* NC = Negative control; M = Ladder; PC = Positive control

4.2 Partial gene sequencing

As further confirmation was done through sequencing, based on the sequence comparison with the published sequences from the Genbank database, the findings of the four suspected positive serum samples showed no detectable similarities to any strain of virus from the *Coronaviridae* family (Figure 7). Therefore, all samples of 35 rats were negative for *Coronaviridae* RNA.

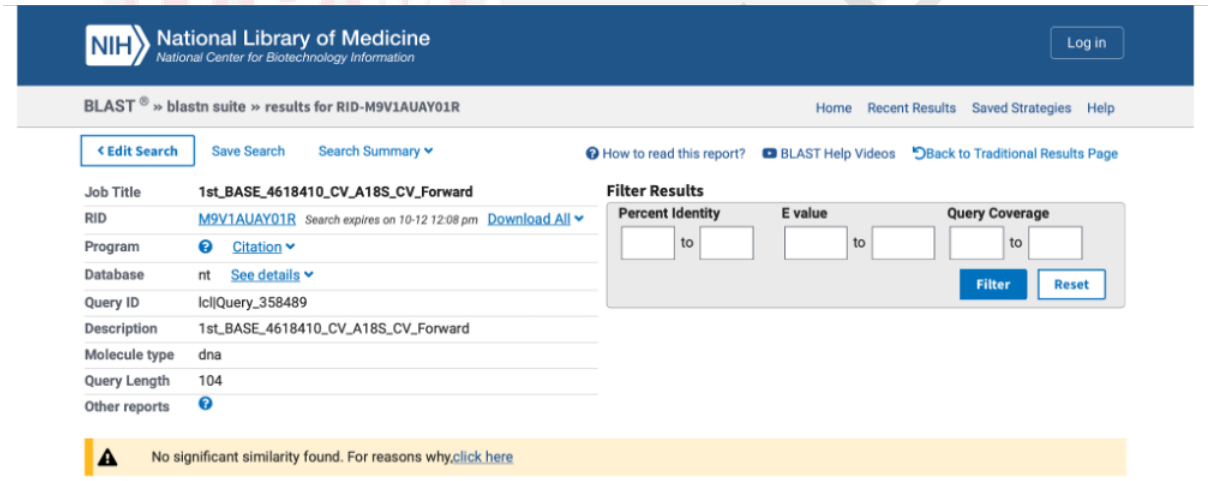



Figure 7. The BLAST analysis of *Coronaviridae* RNA from one of the suspected positive samples. Sequencing based on forward primer shows no significant similarity to the *Coronaviridae* family.

## 5.0 DISCUSSION

In this study, all 35 rats were negative for *Coronaviridae* RNA because there was no detection of *Coronaviridae* RNA in the rats in Klang Valley. The negative results in this study are still in agreement with other studies. The same finding had also been reported, where none of the 39 rats tested positive for SARS-CoV-2 infection due to no exposure to SARS-CoV-2 (Colombo et al., 2020). Similarly, none of the 110 *Rattus* spp. faecal samples were found positive for *Coronaviridae* in another study (Monastiri et al., 2021).

The negative results of *Coronaviridae* in rats in this study could be due to the sample size being too small. A smaller sample size reduced the opportunity to detect the virus infection within a population. In a previous study reported by Wang et al. (2015), where a larger sample size was used than the current study in order to detect coronavirus, they obtained 30 out of 1465 rats that were found positive, which gave the prevalence of only 2% in their study.

Other than that, the importance of the type of samples tested may also play a role in detecting the *Coronaviridae* RNA. In this study, samples of blood serum and oropharyngeal swabs were used to detect *Coronaviridae* in their viraemic state. McIver et al. (2020) stated that the detection of *Coronaviridae* RNA is frequently used in intestine or fecal samples. In addition, another study conducted using intestinal samples found 39 out of 696 (5.6%) rats were positive for *Coronaviridae* (Wang et al., 2020).

Despite the fact that no rats in Klang Valley were found to have *Coronaviridae* RNA in this study, they are still a potential risk to the community due to their close proximity.

The absence of *Coronaviridae* RNA within the population of rats in Klang Valley does not mean that the rats were never exposed to the virus. It is possible that the rats had no current infection, resulting in low levels of antigen in their blood, or that the rats had antibodies against coronavirus that could not be detected using RT-PCR. Therefore, the monitoring and surveillance of *Coronaviridae* in rats in Malaysia still need to be conducted for awareness of the potential zoonotic risk and to improve the prediction of spillover events.

## 6.0 CONCLUSION

According to the results of this study, all 35 rats tested negative for *Coronaviridae* RNA. Therefore, there is no detection of *Coronaviridae* RNA in the population of *Rattus spp.* in Klang Valley due to no significant similarity to any strain of virus from the family of *Coronaviridae* that was revealed from the partial gene sequencing analysis of the samples.

## 7.0 RECOMMENDATIONS

In order to improve for further study, it is recommended to increase the sample size to enhance the chance of getting more positive results, as many studies done previously to detect *Coronaviridae* that have shown positive results used a larger sample size. Other than that, expansion of sample types other than serum and oropharyngeal swabs shall be conducted. For example, intestinal samples used in a study from China revealed positive results for rodent coronavirus enteric tropism. A serological test is also recommended in further studies to detect the presence of antibodies, as detecting the presence of antigen might not be accurate as there is no current infection going on.

REFERENCES

Bartak, M., Słońska, A., Bańbura, M. W., & Cymerys, J. (2021). SDAV, the rat coronavirus—How much do we know about it in the light of potential zoonoses. *Viruses,* 13(10). https://doi.org/10.3390/v13101995

Bhatt, P. N., Percy, D. H. and Jonas, A. M. (1972). Characterization of the Virus of Sialodacryoadenitis of Rats: A Member of the Coronavirus Group. *Journal of Infectious Diseases,* 126(2), 123–130.

Bovo, D., Rugge, M., & Shiao, Y. H. (1999). Origin of spurious multiple bands in the amplification of microsatellite sequences. *Molecular Pathology*, *52*(1), 50. https://doi.org/10.1136/MP.52.1.50

Camp, J. V., Desvars-Larrive, A., Nowotny, N., & Walzer, C. (2022). Monitoring Urban Zoonotic Virus Activity: Are City Rats a Promising Surveillance Tool for Emerging Viruses?. *Viruses*, *14*(7). https://doi.org/10.3390/v14071516

Colombo, V. C., Sluydts, V., Mariën, J., vanden Broecke, B., van Houtte, N., Leirs, W., Jacobs, L., Iserbyt, A., Hubert, M., Heyndrickx, L., Goris, H., Delputte, P., de Roeck, N., Elst, J., Ariën, K. K., Leirs, H., & Gryseels, S. (2022). SARS-CoV-2 surveillance in Norway rats (*Rattus norvegicus*) from Antwerp sewer system, Belgium. *Transboundary and Emerging Diseases*, *69*(5), 3016–3021. https://doi.org/10.1111/tbed.14219

Hartley, J. W., Rowe, W. P., Bloom, H. H. and Turner, H. C. (1964). Antibodies to Mouse Hepatitis Viruses in Human Sera. Experimental Biology and Medicine, 115(2), 414–418.

Huong, N. Q., Nga, N. T. T., Van Long, N., Luu, B. D., Latinne, A., Pruvot, M., Phuong, N. T., Quang, L. T. V., Van Hung, V., Lan, N. T., Hoa, N. T., Minh, P. Q., Diep, N. T., Tung, N., Ky, V. D., Roberton, S. I., Thuy, H. B., Van Long, N., Gilbert, M., Wicker, L., Mazet, J. A. K., Johnson, C.K., Goldstein, T., Tremeau-Bravard, A., Ontiveros, V., Joly, D. O., Walzer, C., Fine, A. E. & Olson, S. H. (2020). Coronavirus testing indicates transmission risk increases along wildlife supply chains for human consumption in Viet Nam, 2013-2014. *PLOS ONE*, *15*(8). https://doi.org/10.1371/JOURNAL.PONE.0237129

Jonas, A M.; Craft, J; Black, L; Bhatt, P N.; and Hilding, D, (1969). Sialodacryoadenitis in the rat. A light and electron microscopic study. Faculty Research 1960 - 1969. 776.

Lau, S. K. P., Woo, P. C. Y., Li, K. S. M., Tsang, A. K. L., Fan, R. Y. Y., Luk, H. K. H., Cai, J., Chan, K., Zheng, B.-J., Wang, M., & Yuen, K. (2015). Discovery of a Novel Coronavirus, China *Rattus* Coronavirus HKU24, from Norway Rats Supports the Murine Origin of *Betacoronavirus 1* and Has Implications for the Ancestor of *Betacoronavirus* Lineage A. *Journal of Virology*, *89*(6), 3076–3092. https://doi.org/10.1128/jvi.02420-14

Maclachlan, N.J., Dubovi, E. J., Barthold, S. W., Swayne, D. E. and Winton, J. R. (2016). Chapter 24 - *Coronaviridae*. *Fenner's Veterinary Virology. 5th ed.*, 435-461.

Malik, Y. A. (2020). Properties of Coronavirus and SARS-CoV-2. *Malaysian J Pathol,* 42(1), 3-11. Retrieved from http://www.mjpath.org.my/2020/v42n1/properties-of-coronavirus.pdf

McIver, D. J., Silithammavong, S., Theppangna, W., Gillis, A., Douangngeun, B., Khammavong, K., Singhalath, S., Duong, V., Buchy, P., Olson, S. H., Keatts, L., Fine, A. E., Greatorex, Z., Gilbert, M., LeBreton, M., Saylors, K., Joly, D. O., Rubin, E. M., & Lange, C. E. (2020). Coronavirus surveillance of wildlife in the Lao People's Democratic Republic detects viral RNA in rodents. *Archives of Virology*, *165*(8), 1869–1875. https://doi.org/10.1007/s00705-020-04683-7

Meerburg, B. G., Singleton, G. R., & Kijlstra, A. (2009). Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology,* 35(3), 221–270. https://doi.org/10.1080/10408410902989837

Mohd-Qawiem, F., Nur-Fazila, S.H., Ain-Fatin, R., Yong, Q.H., Nur-Mahiza, M.I., & Yasmin, A.R. (2022). Detection of zoonotic-borne parasites in *Rattus* spp. in Klang Valley, Malaysia. *Veterinary World,* 15(4), 1006-1014.

Monastiri, A., Martín-Carrillo, N., Foronda, P., Izquierdo-Rodríguez, E., Feliu, C., López-Roig, M., Miquel, J., Ar Gouilh, M., & Serra-Cobo, J. (2021). First Coronavirus Active Survey in Rodents From the Canary Islands. *Frontiers in Veterinary Science*, *8*. https://doi.org/10.3389/fvets.2021.708079

Nova, N. (2021). Cross-Species Transmission of Coronaviruses in Humans and Domestic Mammals, What Are the Ecological Mechanisms Driving Transmission, Spillover, and Disease Emergence? *Frontiers in Public Health, 9*(717941). https://doi.org/10.3389/fpubh.2021.717941

Otto, G. M., Franklin, C. L., & Clifford, C. B. (2015). Chapter 4 - Biology and Diseases of Rats. *Laboratory Animal Medicine: Third Edition*, 151–207. https://doi.org/10.1016/B978-0-12-409527-4.00004-3

Parker, J. C., Cross, S. S. and Rowe, W. P. (1970). Rat coronavirus (RCV): A prevalent, naturally occurring pneumotropic virus of rats. *Arch Gesamte Virusforsch,* 31(3), 293–302.

Payne, S. (2017). Family *Coronaviridae*. *Viruses: From Understanding to Investigation,* 149–158. https://doi.org/10.1016/b978-0-12-803109-4.00017-9

Robinson, S. J., Kotwa, J. D., Jeeves, S. P., Himsworth, C. G., Pearl, D. L., Weese, J. S., Lindsay, L. R., Dibernardo, A., Toledo, N. P. L., Pickering, B. S., Goolia, M., Chee, H. Y., Blais-Savoie, J., Chien, E., Yim, W., Yip, L., Mubareka, S., & Jardine, C.M. (2022). Surveillance for SARS-CoV-2 in Norway rats (*Rattus norvegicus*) from southern Ontario. https://doi.org/10.22541/au.166214344.47276029/v1

Wang, W., Lin, X. D., Guo, W. P., Zhou, R. H., Wang, M. R., Wang, C. Q., Ge, S., Mei, S. H., Li, M. H., Shi, M., Holmes, E. C., & Zhang, Y. Z. (2015). Discovery, diversity and evolution of novel coronaviruses sampled from rodents in China. *Virology*, *474*, 19–27. https://doi.org/10.1016/j.virol.2014.10.017

Wang, W., Lin, X. D., Zhang, H. L., Wang, M. R., Guan, X. Q., Holmes, E. C., & Zhang, Y. Z. (2020). Extensive genetic diversity and host range of rodent-borne coronaviruses. *Virus Evolution*, *6*(2). https://doi.org/10.1093/ve/veaa078

Wasberg, A., Raghwani, J., Li, J., Pettersson, J. H. O., Lindahl, J. F., Lundkvist, Å., & Ling, J. (2022). Discovery of a Novel Coronavirus in Swedish Bank Voles (*Myodes glareolus*). *Viruses*, *14*(6). https://doi.org/10.3390/v14061205

Watanabe, S., Masangkay, J. S., Nagata, N., Morikawa, S., Mizutani, T., Fukushi, S., Alviola, P., Omatsu, T., Ueda, N., Iha, K., Taniguchi, S., Fujii, H., Tsuda, S., Endoh, M., Kato, K., Tohya, Y., Kyuwa, S., Yoshikawa, Y., & Akashi, H. (2010). Bat Coronaviruses and Experimental Infection of Bats, the Philippines. *Emerging Infectious Diseases*, *16*(8), 1217-1223. https://doi.org/10.3201/EID1608.100208

## APPENDICES

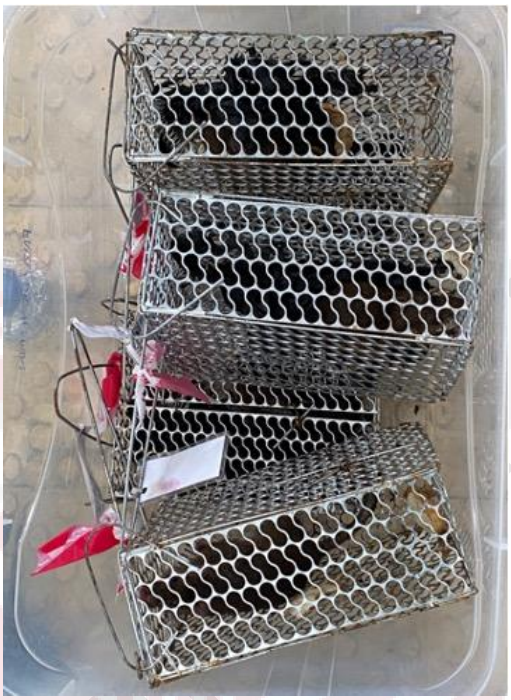
Appendix 1. The usage of personal protective equipment during sampling.

Appendix 2. Cages that were used to trap the rats in the markets.

Appendix 3. Baits that were prepared to attract the rats into the cage trap.

Appendix 4. Placement of traps around the market areas.

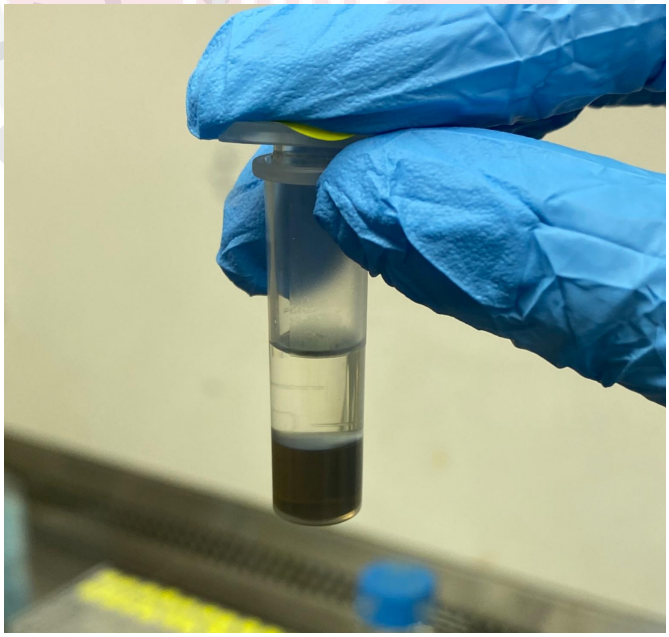
Appendix 5. Anaesthesia of rats using chloroform was done by trapping in a container box.



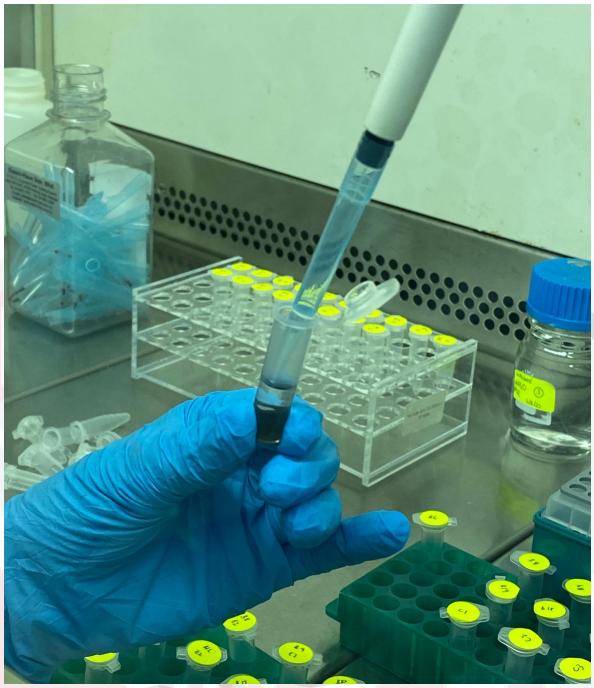
Appendix 6. Collection of blood via intracardiac puncture.

Appendix 7. Collection of oropharyngeal swab using sterile cotton swab.

Appendix 8. The separation phase of RNA extraction shows 3 layers.



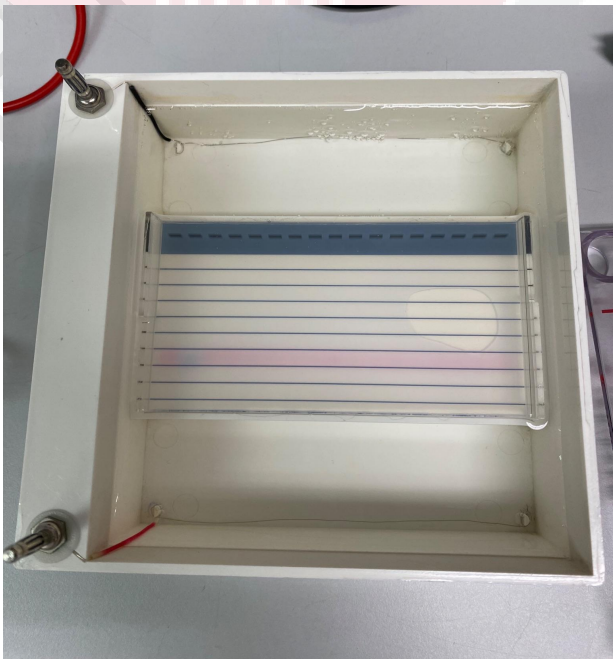
Appendix 9. The removal of aqueous layer for RNA precipitation with isopropyl alcohol.



Appendix 10. The removal of supernatant and washing RNA pellet with ethanol.

Appendix 11. Air drying the pellet followed by redissolving the RNA with sterile $ddH_2O$.

Appendix 12. Gel electrophoresis using 2.0% agarose gel ran with 1x TAE buffer to visualise the PCR products.



Appendix 13. Preparation of partial gene sequencing sample was done by attaching the PCR tubes to the card provided by the company.