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DETECTION OF BUNYAVIRIDAE VIRUS RNA AMONG RATTUS SPECIES IN KLANG VALLEY BY MOLECULAR METHOD

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DETECTION OF *BUNYAVIRIDAE* VIRUS RNA AMONG *RATTUS* SPECIES IN KLANG VALLEY BY MOLECULAR METHOD

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A project paper submitted to the
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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Detection of *Bunyaviridae* virus RNA among *Rattus* species in Klang Valley by molecular method", by Nur Aliah Batriesha binti Eleshamsor, and in our opinion it is

satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

This project paper is dedicated to Allah S.W.T., who had created me
and made all circumstances possible throughout this project
with His guidance and love.

To my family,

abah, Eleshamsor bin Ilias

mama, Noraini bt Mohd Salleh,

abang & adik;
Naief Iman, Alif Hazmie

My friends,

and always, my Aiman

I sincerely thank you for your endless care and support, mentally and physically.
May there be blessings and success in your life.

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Praise to Allah the Almighty for being granted physical and mental strength throughout my life, for desiring knowledge for self-improvement and contribution for my country in the economic and academic field to become more successful moving forward.

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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 *BUNYAVIRIDAE* DALAM KALANGAN SPESIES *RATTUS* DI LEMBAH KLANG MENGIKUT KAEDAH MOLEKUL

Oleh

NUR ALIAH BATRIESHA BINTI ELESHAMSOR

2022

Penyelia: Nor Yasmin Abd Rahaman

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Di Asia dan Eropah, hantavirus daripada keluarga *Bunyaviridae* adalah virus zoonotik bawaan tikus yang boleh menyebabkan demam berdarah dengan sindrom buah pinggang (HFRS) pada manusia.Sesetengah spesies tikus bertindak sebagai perumah semula jadi hantavirus yang menjangkiti manusia melalui penyedutan air kencing, najis, air liur dan gigitan aerosol. Kajian serologi jangkitan hantavirus dalam sampel tikus sebelum ini telah dijalankan pada tahun 2002

dan mendedahkan bahawa 2.52% manusia dan 15.91% tikus didapati positif hantavirus di Pelabuhan Klang dan Kelantan, Malaysia. Walau bagaimanapun, kajian terkini mengenai prevalen molekul virus ini di Malaysia adalah terhad. Oleh itu, kajian ini bertujuan untuk mengesan virus *Bunyaviridae* dalam *Rattus* spp. di Lembah Klang, Malaysia melalui kaedah molekul. Sampel serum darah dan calit orofarinks yang dikumpul daripada tikus liar (n=35) yang diperangkap dari tiga tapak kajian dalam Lembah Klang telah dianalisis dengan pembalikkan transkriptase-tindak balas rantai polimerase (RT-PCR) yang menyasarkan gen S. Produk RT-PCR seterusnya dianalisis dengan gel elektroforesis menggunakan gel agarose 2% dimana amplikon bersaiz 930 bp dijangka daripada ujian tersebut. Satu sampel yang menunjukkan jalur yang hampir sama dengan saiz amplikon yang disasarkan dianggap sebagai sampel positif dan telah dihantar untuk penjujukan DNA separa. Analisis RT-PCR dan penjujukan DNA menunjukkan bahawa tiada RNA *Bunyaviridae* didapati dalam kesemua 70 sampel yang diuji. Dapatan awal ini mencadangkan bahawa tikus daripada kajian ini tidak dijangkiti hantavirus *Bunyaviridae*. Walau bagaimanapun, saiz sampel yang kecil mungkin telah mengaburi kelaziman sebenar pengesanan virus ini, justeru kajian lanjut diperlukan untuk membongkar kesamaran tersebut.

Kata kunci: *Bunyaviridae; hantavirus***;** zoonotik; tikus liar; RT-PCR

ABSTRACT

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

DETECTION OF *BUNYAVIRIDAE* VIRUS RNA AMONG *RATTUS* SPECIES IN KLANG VALLEY BY MOLECULAR METHOD

By

NUR ALIAH BATRIESHA BINTI ELESHAMSOR

2022

Supervisor: Nor Yasmin Abd Rahaman

Co- Supervisor: Nur Fazila Saulol Hamid and Prof. Dr. Siti Suri Arshad

In Asia and Europe, hantaviruses from the *Bunyaviridae* family are commonly associated with rodent-borne zoonotic viruses that can cause hemorrhagic fever with renal syndrome (HFRS) in humans. Some rat species act as a natural reservoir of hantaviruses that infect humans via inhalation of aerosolized urine, feces, saliva and bites. Previous serological study of hantavirus infection in rat samples was conducted in 2002 and has revealed that 2.52% of humans and 15.91%

of rodents were positive of hantavirus in Port Klang and Kelantan, Malaysia. However, an updated study on the molecular prevalence of this virus in Malaysia is lacking. Therefore, this study was aimed to detect *Bunyaviridae* virus in the *Rattus* spp. in Klang Valley, Malaysia by molecular method. Blood serum and oropharyngeal swabs samples collected from 35 cage-trapped wild rats at three study sites within Klang Valley were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) targeting the S gene. The RT-PCR product was further analyzed by gel electrophoresis using 2% agarose gel and amplicon size of 930 bp was expected from the assay. One sample that showed a band approximate to the targeted amplicon size, regarded as a suspected positive sample, was sent for partial DNA sequencing. The RT -PCR and DNA sequencing analyses showed that *Bunyaviridae* RNA were absent from all 70 tested samples. This preliminary finding suggested that the rats from this study were not infected with *Bunyaviridae* related hantaviruses. However, a small sample size may obscure the true prevalence of the virus detection, therefore further study is needed to unravel the ambiguity.

Keywords: *Bunyaviridae; hantavirus***;** zoonotic; wild rats; RT-PCR

1. INTRODUCTION

Infectious diseases frequently have various intraspecific routes of pathogen transmission within wildlife and other populations (Minter et al. 2017). One of the most notorious wild animals that can contribute to zoonotic disease transmission as they can be the significant reservoir of some zoonotic pathogens are the rats, members of the rodent family *Muridae* (Himsworth et al. 2014). Furthermore, the physiological similarity between rats and humans, their high adaptability, frequency and intensity within urban areas, as well as their natural tendency for close association with humans are factors that highly contribute to zoonotic disease transmission (Himsworth et al. 2013). An understanding of the crucial role of rats in carrying and transmitting diseases within the population is important as it poses a great health risk to the humans and it helps in taking measures in preventing any possible diseases transmission.

Bunyaviridae family is a large viral family that consists of five genera which are Hantavirus , Nairovirus , Orthobunyavirus, Phlebovirus and Tospovirus. Viruses in the *Bunyaviridae* family are widely distributed throughout the world and have been known to infect invertebrates, vertebrates, and plants, exemplifying the family's great diversity. Bunyaviruses have a tripartite single-stranded negative-sense RNA genomes which are segment S, segment M and segment L that encodes four structural proteins and one or two additional nonstructural proteins.

Indeed, most viruses of the family are arthropod-borne but some of them can be transmitted to small mammals which can potentially cause significant zoonotic diseases including encephalitides and viral hemorrhagic fevers in humans. Basically, Sandfly Fever Sicilian Virus (SFSV), Crimean-Congo Hemorrhagic Fever Virus (CCHFV), La Crosse Virus (LACV) which are caused by

Phlebovirus, Nairovirus and Orthobunyavirus respectively are the examples of the arthropod-borne diseases that can significantly transmitted to humans. On the other hand, one of the member of *Bunyaviridae* family, hantavirus are rodent-borne viruses that can cause two important zoonotic diseases which are haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) in humans. In addition to hantavirus, a relatively diverse common host range that live in close proximity within human population such as rodents, shrews, moles, and bats can be the carrier of these important human pathogenic hantaviruses which can be the concerning threat to public health (Munir et al. 2020).

In Malaysia, there is a lack of studies on the epidemiology of *Bunyaviridae* in wild rats. Only few previous studies were conducted and yet one of them has revealed a less significant number of positive samples for hantavirus which is 16 out of 131 (12.21%) total samples from Kelantan and Port Klang (Lam et al. 2015). The significance of *Bunyaviridae* infections is unaware of and has been treated with low concern. Whereas rats are common rodent species that live in close proximity together within human population, the role of rats as potential hosts and reservoirs of the *Bunyaviridae* should deserve more attention as it can cause zoonotic diseases. This study will serve as an additional database on the status of *Bunyaviridae* in *Rattus* spp. in Klang Valley, Malaysia specifically by detecting the presence of *Bunyaviridae* RNA among the rats in molecular method, reverse transcriptase - polymerase chain reaction (RT-PCR).

The hypothesis in this study is:

1. *Bunyaviridae* is not detected among *Rattus* species in Klang Valley, Malaysia.

Therefore, the objective of this study is :

1. To detect the presence of *Bunyaviridae* RNA among *Rattus* species in Klang Valley, Malaysia.

2. LITERATURE REVIEW

In the late nineteenth century, many bunyavirus diseases were undoubtedly known long before viruses were discovered (Whitehouse et al. 2015). The name of the family comes from the location in Uganda where the prototype bunyavirus was discovered (MacLachlan et al., 2011). The *Bunyaviridae* family, whose members are commonly referred to as bunyaviruses, is the largest and arguably among the most diverse families of RNA viruses (Calisher, 1986; Nichol et al., 2005). The *Bunyaviridae* comprises five genera: Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus, and Tospovirus. Classification within these genera was initially based on serologic cross-reactivity, but has been extended to include genome organization, virion morphology, lifecycles, and phylogenetic relationships (Porterfield, 1975; Schmaljohn, 1996; Nichol et al., 2005).

2.1 Classification

Viruses from three genera, Orthobunyavirus, Nairovirus, and Phlebovirus, remain viable in arthropod-vertebrate-arthropod cycles with specificity for both arthropod vectors and vertebrate reservoir hosts. This specificity is the basis for each virus's typically narrow geographic and ecological niches (MacLachlan et al., 2011). Viruses in the genus Hantavirus are distinguishable among bunyaviruses in that they can be transmitted by exposure to virus-contaminated secretions or excretions from their rodent, shrew, or mole hosts rather than arthropod vectors (Elliott, 1997; Kang et al. 2011).

Table 1. Representative Genus of the Bunyaviridae associated with diseases of vertebrates

Genus	Virus	Reservoir hosts/ vector	Distribution	Significant diseases in animals and human	References
Orthobunyavirus	La Crosse virus (LACV) Cache Valley virus (CVV)	Arthropod Culicoides spp. (Aedes species, Culiseta inornata, Sandflies)	North America	Human (encephalitis and aseptic meningitis) Domestic animals (sheep and cattle)	Whitehouse et al. 2015
	Akabane virus		Africa, Asia		
Phlebovirus	Rift Valley Virus	Arthropod-mosquitoes (Aedes spp., Culex spp.)	Africa, Madagascar, Egypt, Saudi Arabia	Sheep, camels, goats, cattle, and Asian water buffalo Human(Hepatitis, haemorrhagic disease, retinitis, meningoencephalitis)	Soldan et al. 2014 Whitehouse et al. 2015

Nairovirus	Nairobi Sheep Disease	Arthropod-Ixodid Ticks (Rhipicephalus appendiculatus. culicoid flies	Central and East Africa, Asia, Eastern Europe	Sheep and goats (Hemorrhagic gastroenteritis, abortion, 75% mortality in sheep)	Leger et al. 2015
	Crimean Congo hemorrhagic fever viruses (CCHFV)			Human (Severe haemorrhagic disease)	
Hantavirus	Hemorrhagic fever with renal syndrome (HFRS)	Rodents- Small mammals (rats, bats, moles)	Asia , Europe	Rodents/ small mammals (rats, deer mice, shrew, and moles)	Mattar et al. 2015
	Hantavirus pulmonary syndrome (HPS)		America	Human	Munir et al. 2015 MacLachlan et al. 2011

2.2 Pathogenesis

Each protein coded aids in viral replication strategies in such ways as host cell attachment by cellular membrane fusion, RNA protection from degradation process and the RNA-dependent RNA polymerase activity possession in host cells. Basically, after viral glycoproteins, Gn and Gc are attached to the host cells by cell surface lectin receptors (Leger et al. 2015), the fusion with cellular membranes allows the viral entry and followed by endocytosis. The replication of the virus occurs in the cytoplasm followed by virus congregation and maturation which is localized to the Golgi apparatus. Mature virions are then transported to the plasma membrane in small intracytoplasmic vesicles after budding into the Golgi cisternae. Virion release occurs after vesicle fusion with the plasma membrane throughout the cytoplasm, in a manner similar to normal exocytosis (Whitehouse et al. 2015). However, even though the viruses possess the same replication mechanism, distinctions between the genera are based on antigenic, serological, molecular, structural differences, natural hosts and transmission cycles (Calisher 1996; Soldan et al. 2005; Hardestam et al. 2007). Notably, each of the five identified genera of bunyaviruses possesses members that have a negative impact on human health, well-being, or economic development including for the case of viruses in the genus Tospovirus, it can either directly causing human disease or indirectly causing damage to domestic animals or plant crops (Nichol et al., 2000; Soldan and Gonzalez-Scarano, 2005; Elliott, 2009).

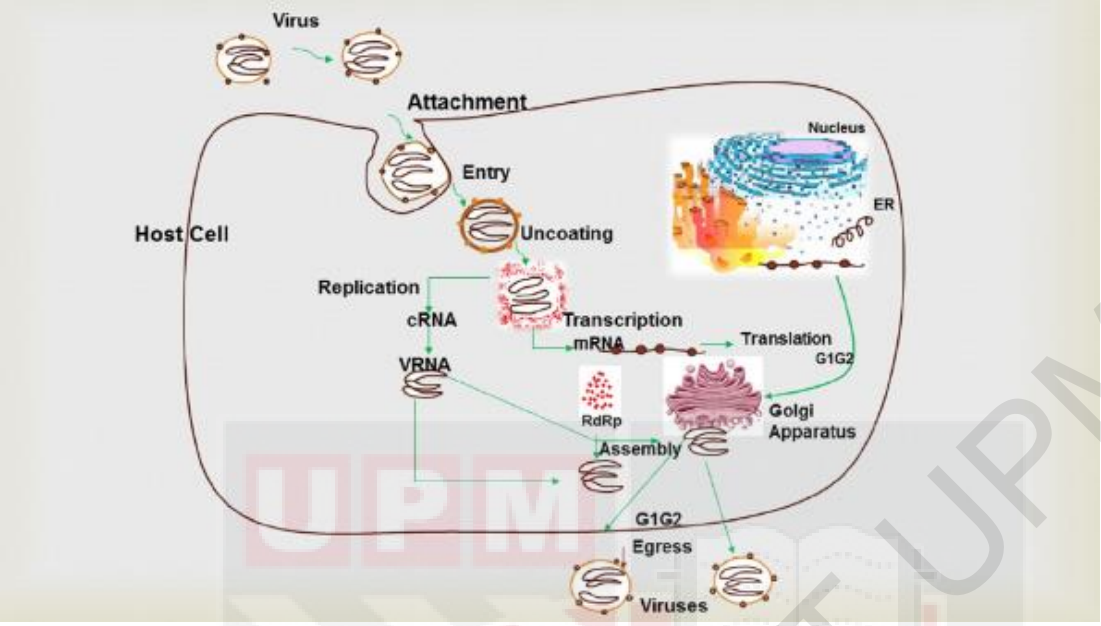

Figure 1. Hantavirus replication cycle main step started by the attachment with virus molecules to the cell's surface between viral glycoprotein and the host's cell surface receptors (Munir et al. 2019).

2.3 Manifestation of Infection

Except for Tospovirus, which is a plant-infecting virus, bunyaviruses are divided into arthropod-borne and rodent-borne viruses. These bunyavirus features are related to the nature of the virions, their biological properties, and their transmission cycles in natural hosts (MacLachlan et al., 2011). The differences between viruses carried by arthropods and rodents are based on the exposure ability of the virus to an environment outside the host. The arthropod-borne viruses are unlikely to be exposed to an environment other than the host compared to the rodent-borne virus; hantavirus which are more stabilize in an environment other than the host as the transmission occurs through wounding or inhalation of aerosolized virus-contaminated excreta in the environment (Hardestam et al. 2007). Bunyaviruses cause life-long latent infection in their reservoir hosts, resulting in

asymptomatic virus shedding or clinical signs, allowing the virus to spread ubiquitously in the rat population's enclosed environment (Barthold et al. 2016). Human disease caused by bunyavirus infection ranges from mild to severe, including pulmonary disease, hemorrhagic fever, meningitis, and encephalitis (Soldan et al., 2014).

Hantaviruses are rodent-borne zoonotic viruses that cause two major clinical syndromes in humans: hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe, and hantavirus pulmonary syndrome (HPS) in the Americas (Mattar et al. 2015). Hantavirus differs significantly from other members of the family in several ways, including the fact that it cannot be transmitted through arthropods as other members do (Munir et al. 2015). They are transmitted among rodents through long-term shedding of saliva, urine, and faeces (MacLachlan et al. 2011). In most cases, hantavirus infection is transferred to humans through the inhalation of aerosolized rodent urine, faeces, or saliva. Animal bites have also been linked to infection (Hartline et al. 2013). In Southeast Asia, hantavirus infection is not given the attention it deserves (Lam et al. 2002). Previously, hantavirus was found in rodent populations in Peninsular Malaysia, Thailand, and Singapore (Lim et al. 1985; Wong et al. 1985; Nitatpattana et al. 2000; Lam et al. 2001). There have only been three reports of hantavirus infections in humans in Malaysia. The present study supports previous reports of hantavirus infections in humans and rodents in Malaysia (Lam et al. 2002).

Rats (*Rattus* spp.) are a reservoir for a variety of zoonotic pathogens that cause significant human morbidity and mortality. There are commonly multiple potential routes of intraspecific pathogen transmission within wildlife and other populations (Himsworth et al. 2013). Zoonotic diseases are caused by pathogens that spread from nonhuman animals to humans via a process known as zoonotic spillover (Ellwanger et al. 2021). These diseases, particularly those associated with wild animals, pose a significant threat to human health. The presence and severity of infection in

reservoir hosts, which are typically non-human animals from wild environments or farms, will have the greatest influence on the risks of a spillover event occurring. The distribution and density of infected hosts in a given environment are also important factors in determining spillover risk (Plowright et al., 2017) In other words, urban areas are of significant concern for the emergence of zoonotic diseases because they provide ideal habitat for certain wild species, resulting in increased contact with humans and, potentially, zoonotic disease transmission (Cleveland et al. 2001; Daszak et al. 2000). *Rattus* species are perhaps the most notorious of the wild animals found in cities, due to the frequency and severity with which they infest urban areas, as well as their proclivity for close association with people. In this sense, as interactions between humans and other animal species get more intense and frequent, the chances of new pathogens being introduced into the human population increase (Ellwanger et al. 2021).

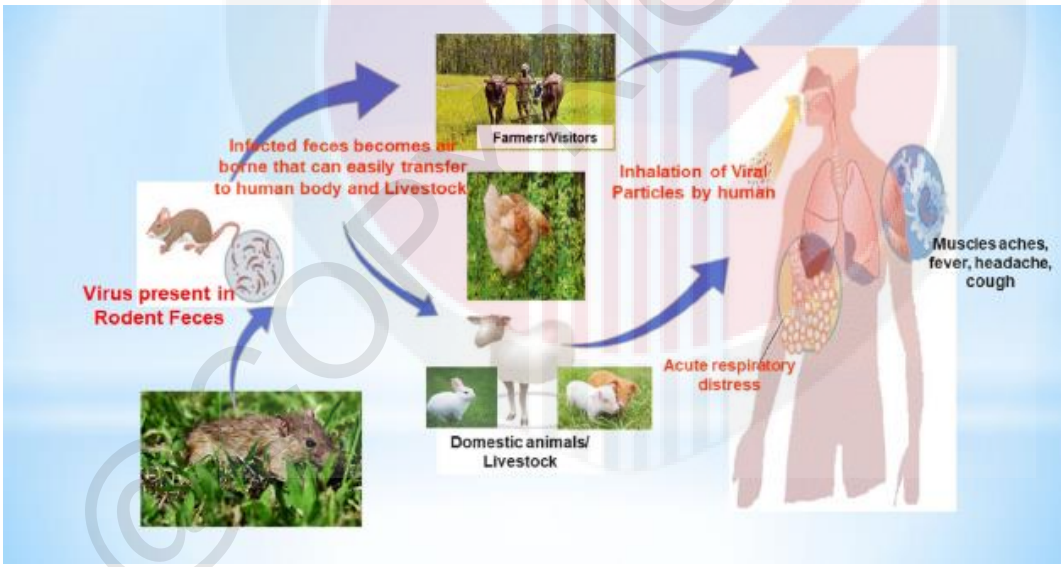

Figure 2. Hantavirus transmission occurs by inward breath of aerosolized viral particles in the urine, defecation, and salivation discharged into the environment Retrieved from Munir et al. 2019.

2.4 Diagnosis

2.4.1. Isolation in cell culture

Due to their broad spectrum of permeability and relative ease of maintenance in the laboratory, cell cultures are the most commonly used host system for virus cultivation (Leland et al. 1988). Virus isolation from human samples is uncommon, it is not a diagnostic option for human hantavirus infection. However, hantavirus isolation for research purposes can be accomplished using clinical materials inoculated onto Vero E-6 cell cultures. Immunofluorescence can be used to identify hantavirus-infected cells one to two weeks after inoculation. Isolation is time-consuming and labor-intensive, poses a high risk of viral contamination in the laboratory, and necessitates biosecurity measures to protect the handler from aerosol exposure (Mattar et al.2015).

2.4.2. Serology method

In today's practice, serological tests commonly used include enzyme-linked immunosorbent assays (ELISA), immunofluorescence assays (IFA), and reduction neutralization tests (Vainionpaa et al. 2008). Basically, the presence of specific IgM antibodies or a notable increase in the levels of specific IgG antibodies are demonstrated between two consecutive samples which are taken seven to ten days apart. The presence of this immunogenic component in the body indicates a result of pathogen infection due its defensive action based on their binding to the antigens (Regenmortel et al 1981). The viral structural proteins such as Gn, Gc, and N protein can stimulate a high level of IgM antibodies, which can be discovered at the onset of symptoms (Mattar et al.2015). The virus-specific antibody titers reach detection levels when immunoglobulin M (IgM) antibodies are produced followed by immunoglobulin E (IgE) antibodies a few days later (Hampton et al. 1990). The test should be confirmed with increased presence of IgG or IgM titers in 2–4 weeks. Hence,

antibodies are typically discovered in serum samples collected during the acute phases of an infection (Vainionpaa et al. 2008). In serological tests, the titre of a specific antibody refers to the relative concentration of antibodies in the serum which is based on its greatest dilution when reacting with its homologous antigen. The titre is defined as the reciprocal of the highest dilution factor that causes a serological reaction (Hampton et al. 1990). Some limitations for serological tests are when the antibody response is insufficient, or the assay's limited specificity, it prevents clear interpretation of the results as well as in immunocompromised patients where the serological response is commonly too weak to demonstrate specific responses (Leinikki et al. 2008).

2.4.3. Molecular method

RT- PCR using blood samples is only useful in the early stages of infection when patients are viremic. The RT- PCR product can be sequenced to identify the virus and perform phylogenetic analysis, which is a distinct advantage of PCR (Jahangeer et al. 2019). Moreover, the advantage of PCR to identify the hantavirus RNA is that it could be able to detect the infectious agent even within seven to 10 days after onset of symptoms and it requires about 24 hours to give results (Mattar et al.2015). As a matter of fact, such methods are incredibly useful in identifying viruses that have a high risk of causing fatal conditions even within a few hours. RT-PCR has also been used to diagnose hantavirus infections, with primers specific for the virus.

2.5 Control and Prevention

Overall,the increased habitat available for urban rats and increased contact between rats and disadvantaged people, urban rats can pose a significant health risk to people through the transmission of zoonotic diseases likely in the environment as cities which are more densely populated and increase urban poverty. Understanding a complete and comprehensive knowledge

of urban-rat-associated zoonoses, effective strategies to monitor and eliminate those risks can be applied (Himsworth et al. 2013). This strategy should start with attempting to understand the ecology of the animal reservoir host and identifying the zoonotic pathogens circulating in host populations. The frequency and distribution of RAZ have changed over time as human populations have changed. RAZ has emerged, or re-emerged, in urban areas as a result of increasing urbanization and urban poverty(Himsworth 2014)

Based on the One Health perspective, it is possible to list some actions to prevent spillover events by taking into account the factors that increase or decrease the risks of pathogen transfer between species, as well as the lessons learned from previous outbreaks, epidemics, and pandemics (Ellwanger and Chies, 2018; Ellwanger et al., 2020). In a nutshell, the actions that can be taken are by increasing pathogen surveillance at human-animal interfaces, reducing deforestation and biodiversity loss, avoiding building human housing in forest areas, improving basic/environmental sanitation infrastructure as an initiative to control vectors and free-roaming animals. From the point of view for professionals, Increase investments in human training and laboratories dedicated to the discovery of new pathogens and emerging diseases, as well as the development of vaccines. The biological and social factors that contribute to infection susceptibility should be determined (Ellwanger and Chies, 2018; Ellwanger et al., 2020).

According to the history of health crises, funds and health actions focused on outbreaks and epidemics tend to vanish after such public health emergencies end (Ventura et al., 2020). As a result, actions aimed at preventing spillover events must be implemented on a consistent and systematic basis, with the goal of realistically preventing the emergence of new epidemics. It can be applied by creating financial funds to mitigate the emergence of infectious disease outbreaks from the following spillover events (Ellwanger et al., 2020). Finally, it is essential that the

prevention and mitigation of public health emergencies take into consideration the social, political, and economic aspects of each population (Ventura et al., 2020).

3. MATERIALS AND METHOD

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. Rats trapping and sample collection

Thirty five rats (n=35) were captured randomly in three areas located within Klang Valley which are Pasar Sri Serdang, Pasar Pudu, and Pasar Borong Selangor using 12 cm x 6.5cm x 5cm wire mesh cage traps. The captured rats were numbered sequentially based on the amount of the cages used. Total of 35 wire mesh cage traps were used. Cages were tagged to notify the public the usage of the cages is under a project. Baits such as coconut flesh, white bread and salted fish were used in the cage traps. The baited traps were set in a range of time within 32,400 seconds between 1800 in the evening until 0300 in the morning where the cages were inspected at midnight and taken at 0300 the following morning. The trapped rats (n = 35) were brought to the Post Mortem Laboratory, Faculty of Veterinary Medicine (FPV), UPM, for sampling purposes. The rats were placed in two container boxes and were anesthetized with chloroform (Fisher Scientific, USA) at 100 mg/mL soaked in cotton wool. Average of three to five minutes was taken for the rats to be passed out and anesthetized.

Blood collection was done by intracardiac puncture, followed by cervical dislocation for euthanasia (Mohd-Qawiem et al., 2022). The intracardiac puncture was performed in lateral recumbency as the 21G needle (Terumo, Tokyo, Japan) together with 5 ml syringe (Terumo, Tokyo, Japan) was inserted perpendicular at the point of elbow joint together with the loudest point

of heartbeat palpated to be the landmark of the puncture. Up to 15 mL of blood was collected intracardially in a single rat. The blood samples were processed to obtain serum in the Laboratory of Veterinary Virology, FPV. The blood samples were incubated for 24 hours at room temperature and centrifuged at 1,000 x g (Thermo Fisher Scientific, Waltham, USA) for 10 minutes. Three layers were formed after the centrifugation procedure which are the red blood cells, the buffy coat consists of white blood cells and the plasma layer. Serum samples were taken by plasma layer extraction using a pipette (DLAB, Malaysia) with 1000 microliter pipette tips (Eppendorf, Germany). The serum samples were transferred to 1.5 mL tubes (Eppendorf, Germany) before being stored in the -80°C freezer (SANYO Ultra Low, Japan). Sterile cotton swab tube (BD Vacutainer®, New Jersey, USA) and Phosphate Buffer Solution were used for oropharyngeal swab sampling. The cotton swab was inserted from the mouth until it reached the oropharyngeal area and the samples were mixed together with 2000 μL volume of PBS in the tube. The oropharyngeal swab samples were stored in the -80°C freezer (SANYO Ultra Low, Japan) for further molecular analysis.

Morphometric identification was done where details measurements for body weight, body height, length of ears,tail and hind foot, sex, age categories; juvenile and adult, colour of fur were recorded. Younger rats have fluffier and darker fur than adults and can be distinguished by their fresh, unworn teeth that have not fully erupted from the gums (Phillipps et al. 2018). Some unique characteristics such as number of nipples, number of teeths, and color of the ventral part of the body were also required for precise identification. The species, age, and sex of the rats are recorded in Table 2.

Table 2. Number of rats captured relative to the species, sex, and age

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

3.3. Molecular analysis

3.3.1 RNA extraction

RNA extraction was performed from the serum and oropharyngeal swab samples using TRIsure™ (Bioline, United Kingdom) according to the manufacturer's instructions. The RNA was extracted in a 20 µL sterile autoclaved ddH^2O and was used as the template for reverse transcription-PCR (RT-PCR). RNA purity and concentration were determined using the BioPhotometer (Eppendorf, Germany) at the absorbance value of 260/280 (Eppendorf, Germany). The value of 1.8 to 2.0 indicates a good purity of nucleic acid.

3.3.2 Plasmid and primers

Detection of Bunyavirus was done by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using primers targeting the highly conserved region which is S segments of Bunyavirus (Table 3). A synthetic plasmid (UC2) was used as the positive control with the addition of 20 µL

master mix (Bioline, United Kingdom). Sequences of the primers used in this molecular analysis were as follows: forward primer BUNS-5-U-6 (CGGCGCCAGTAGTGTACTCCAC); and reverse primer BUNS-3-L947 (GCGGCCAGTAGTGTGCTCCAC) (Briese et al. 2007).

Table 3. Forward and reverse primers used in RT-PCR for the detection of S segments Bunyavirus

PCR Reaction	Primers	Primer Sequences	Product size (bp)	Reference
One - step RT-PCR	**S**			
	BUNS-5-U-6	CGGCGCCAGTAGTGTACTCCAC	930	Briese et al. 2007
	BUNS-3-L947	GCGGCCAGTAGTGTGCTCCAC		

3.3.3 RT-PCR assay

One-step RT-PCR was performed using MyTaq™ One-Step RT-PCR (Bioline, United Kingdom) in a 25 µL reaction. Ready-to-use MyTaq™ One Step Mix (Bioline, United Kingdom) was used by preparing a master mix reaction of 4.75 µL of ddH_2O, 12.5 µL of MyTaq™ One Step Mix, and 1.0 µL(10 µM) each of the forward and reverse primers, 0.5 µL of RNAse inhibitor and 0.25 µL of RT-enzyme. Each of the reagents are mixed from highest volume to lowest volume in a 1.5 ml tube. The extracted RNA from the oropharyngeal samples were further subjected to one-step RT-PCR using MyTaq One-Step RT-PCR (Bioline, Memphis, USA) in a 25 µL reaction with primers (10 µM) specific for Bunyaviruses. Some of these samples have been frozen and thawed twice or three times, which is remarkable. However, this suggests that hantavirus genome detection via RT-PCR for this type of sample is applicable (Moreli et al. 2004). In RT-PCR, it is desirable to choose

primers that are complementary to gene sequences that are highly conserved among bunyaviruses . In this study prior to diagnosis, the S segment that encodes the viral nucleoprotein was selected as the target gene. Nucleic acid amplification was performed on a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) with the following protocol: reverse transcription step for 20 minutes at 45 °C, then polymerase activation at 95 °C for 1 minute followed by 45 cycles of 30s denaturation at 95 °C, 1 minute annealing at 48 °C and 1 minute extension at 72 °C. The final extension had similar conditions as the extension step except that it took 3 minutes for the final extension period. The amplification volume was performed according to the manufacturer's instruction, with RT-PCR protocols referred from (Briese et al. 2007).

3.3.4 Gel Electrophoresis

Gel electrophoresis was performed to separate the PCR products using 2.0 % gel prepared by mixing 0.6 g of agarose gel powder (1st BASE, Singapore) in 40 mL TAE (Tris-acetate-EDTA) buffer solution and 2 µL of Red Safe™ (Red Safe™, South Korea) for the visualization of amplified RT-PCR products. The 1 kb DNA Ladder (Vivantis, Malaysia) was used as reference in estimating the size of DNA bands by comparing the DNA bands to the closest fragment in the ladder. The gel electrophoresis was set at 90 V and 350 mA for 35 minutes. The DNA fragments were then observed under the UV light transilluminator (Syngene, United Kingdom). The PCR reaction was considered positive when a 930-bp fragment containing the S- segment was amplified and aligned with a positive control band.

3.3.5 Partial DNA Sequencing

Unpurified positive PCR products were subjected to DNA sequencing by Apical Scientific Sdn. Bhd. The nucleotide sequences obtained from the sequencing analysis 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. RESULTS

4.1 RT-PCR assay

A size of 930 bp amplicon of positive control template can be clearly observed in the RT-PCR result indicating the efficacy of BUNS-5-U-6 and BUNS-3-L947 primers which were used in this protocol. RT-PCR screening of Pan-hantavirus primers for the S segment from rats serum and oropharyngeal swab sample revealed that one of the blood serum (A18) and oropharyngeal swab sample (A18) of *Rattus norvegicus* from Pasar Borong Selangor was suspected to be positive (2.86% (1/35), whereas a faint band approximately appeared in the RT-PCR result and was sent for RNA sequencing. The faint bands are observed and suspected to be at the 930-base pair (bp) compared to the positive control (Figure 1). As for the other samples of serum and oropharyngeal swabs revealed none of the samples of *Rattus norvegicus,* and *Rattus diardii* from Pasar Borong Selangor, Pasar Pudu, and Pasar Seri Serdang were PCR positive for hantavirus RNA (0% (0/35)) (Figure 2).

Figure 3. Gel electrophoresis analysis of Bunyaviruses nucleic acid. A presence of a faint band is observed for samples (A18) at 930 base pair size.

Note NC = Negative control; M = Ladder; PC = Positive control

Figure 4. Absence of any positive bands in samples.

4.2 Nucleotide Sequencing

The suspected RT-PCR positive serum and oropharyngeal swab samples of Rattus Norvegicus species (A18) from Pasar Borong Selangor were selected . Based on the sequence comparison with the published sequences from the GenBank database, the result revealed no significant similarity found to any strain of virus from the *Bunyaviridae* family which indicates negative presence of targeted viral RNA in the sample (Figure 3).

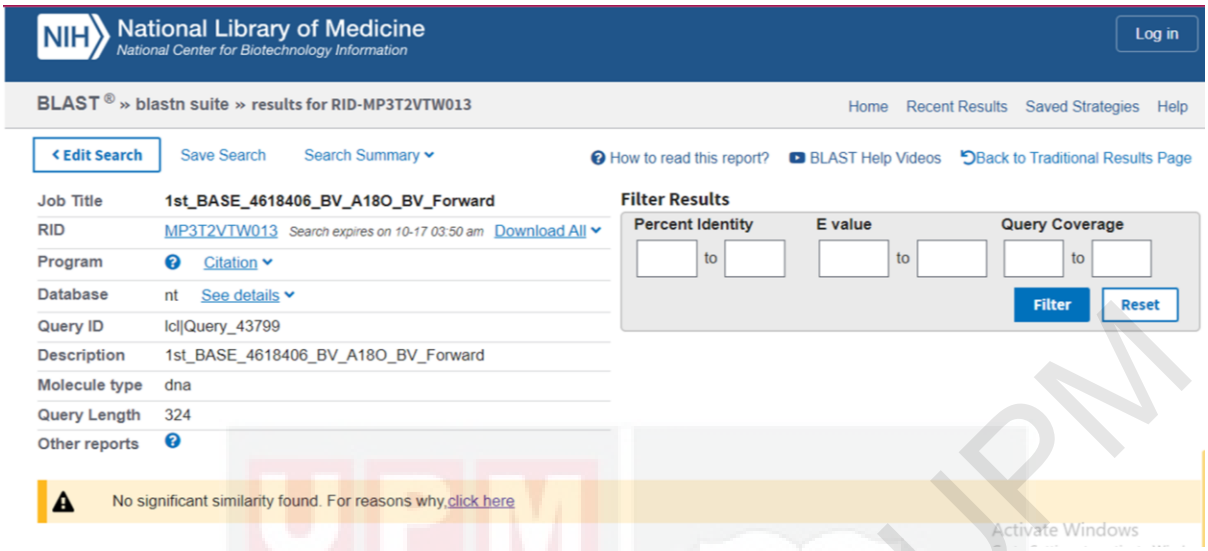

Figure 5. The BLAST analysis of Bunyavirus DNA from one positive isolate. Sequencing based on forward primer shows no significant similarity to the *Bunyaviridae* family.

5. DISCUSSION

Understanding the risk of the virus transmission between different species by crossing of species barriers and the factors that facilitate the virus transmission is essential to establish strategies aimed at reducing the incidence of zoonotic events, reducing the risk of new outbreaks, epidemics, and pandemics.

In this study, detection of bunyaviruses was performed from rodent hosts, *Rattus* species which were majority captured from the wet markets which are considered as the living habitats of these animals due to its high adaptability in the environment where they can thrive for foods, water and protection from fences and walls, rubbish piles and abandoned appliances. Markets contribute significantly to the interaction of humans with different species and new pathogens (Ellwanger et al. 2021). The exposures between humans and the infected rodents can be a concerning public health issue because close contact with infected animals could be a common risk factor of transmission as it possibly occurs through inhaling aerosolized contaminated urine, feces and saliva including infecting by biting (Hartline et al. 2013)

RT-PCR is one of the common diagnostic methods in detecting viral RNA by samples. In this study, the serum and oropharyngeal swab samples obtained from captured wild rats in a chosen area were used for bunyavirus genome detection by RT-PCR. This method is useful to be applied in viral RNA detection from samples of whole blood, discharges, and tissues of infected rodents (Hartline et al 2013).

The negative results of Bunyaviruses RT-PCR from this study could be due to the non-viraemic state of the infection. The results indicate the absence of the virus or low viral load in the blood

during the blood samples collection. The blood serum sample used in this experiment is suitable enough to detect *Bunyaviridae* RNA with sufficient viraemic levels as serum or plasma are more standardized samples used for the study of pathogenesis and viral load (Vaherii et al. 2008). However, the extent of viremia and viral RNA varies between HFRS and HCPS and is primarily determined by the hantavirus type (Vapalahti et al. 2008). In general, severe hantavirus infections caused, for example, by the Hantaan, Dobrava, Sin Nombre, or Andes viruses could leads to high RNA viral load (Terajima et al. 1999; Xiao et al. 2006) compared to Puumala virus which cause lower level of viraemia in the mild form of HFRS (Plyusnin et al. 1999). Samples that are collected within the first seven days after the onset of symptoms, the viral RNA in the circulation of HFRS can be detected by RT-PCR (Vaherii et al. 2008). However, the non-viraemic state of infection could be due to persistent presence of the virus in its shedding sites within the host. For example, the hantavirus has a long-term shedding in saliva, urine, and feces (MacLachlan et al. 2011).

It is also suggested that the non-viraemic state of infection has occurred due to the presence of neutralizing antibodies. The rodents were potentially exposed to hantavirus, but the antibody level declined after a few weeks of infection resulting in negative results (Dubovi et al. 2011). The virus can remain in a rodent's body for up to 270 days after infection, despite the presence of IgM and IgG antibodies specific for hantavirus production (Lam et al. 2001).

These negative results also can be indicated as the rodent populations in this region had not previously been exposed to the virus, and they did not have antibody levels above the detection limit at the time blood sampling was performed (Lee et al. 2015). The exposure of the host to the virus can be evidenced through the presence of antibodies formed in the body. Basically, the very

first step in antibody formation is macrophage phagocytosis of the invading viral particle and the presentation of specific viral antigens to B cells in some form. When a B cell comes into contact with an antigen, it binds it with receptors that are complementary to any of the antigenic determinants (epitopes) on that antigen. The B cell responds to antigen-specific and nonspecific signals from helper T cells by dividing and differentiating into antibody-secreting plasma cells which will secrete antibodies into the blood or lymphatic fluid, or remain attached to the surface of lymphocytes (Herrmann, 1988). From the negative results, it might be indicated that the particular receptors of the virus can be detected by the specific Ig class of the antibodies circulated in the body.

Besides, the small sample size used in this study limits the chances in detecting *Bunyaviridae* infection within the population. Nevertheless, although small sample size were used within the rat population from previous studies that were conducted in detecting the infections, positive detection could be achieved. Examples of a result of 39 out of 524 (7.44 %) rodent samples from political districts and cities in Korea, Seoul were tested positive for hantavirus (Lee et al. 1982). In another study, an example of result where 14 out of 910 (1.53 %) rats samples were serologically tested for severe fever with thrombocytopenia syndrome (SFTSV) strains in Hokkaido, Japan (Lundu et al. 2018).

Limitation in this study suggests that samples of *Rattus* species can be taken from more diverse areas such as the forests and paddy fields where wild rats are the inhabitants. A study conducted in September 2014 where house mice and wild mice from the mountainous areas near the villages in the study sites were captured by snap traps. In comparison to the results, several mice captured

from the mountainous regions were reported positive than the mice captured within the villages. One-step SFTSV real-time RT-PCR detected SFTSV genomic RNA only in 2 of the 8 A. agrarius, but not in 40 R. norvegicus and 4 R. losea (Yang et al. 2015).

Moreover, a serological assay should be conducted to confirm the exposure of *Bunyaviridae* infections in the rat population as it can detect the presence of antibody levels within the body. Considering the biohazard associated with virus isolation, serological testing is typically used to assess infection in reservoir rodent hosts (Maclachlan et al. 2011). Positive serological assay could be the evidence of the infection exposure within the host body. For example, hantavirus structural proteins (Gn, Gc, and N) are able to induce a high level of Immunoglobulin M (IgM) antibodies which are recognizable at the onset of symptoms (Mattar et al. 2015). The titre of IgM antibody as a primary response is likely influenced by incubation time, viral load, immunity, and a variety of other factors. The antibodies can be demonstrated by serological diagnosis such as IgM capture enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), immunoblot assay (IBA) and focus reduction neutralization test (FRNT) (Munir et al. 2020).

Nonetheless, *Rattus* spp. in Klang Valley pose a significant risk to the public due to close proximity of rats and communities in urban areas which may increase the incidence of rat-associated zoonoses. Environmental factors such as poor sanitation and crowding might indeed influence zoonotic diseases associated with rats due to impoverished urban populations. As a result, the rat population must still be managed because it can possess diseases that seem to be hazardous to public health.

6. CONCLUSION

According to the study results, there is no bunyavirus circulation in *Rattus* species in Klang Valley due to no significant similarity to any viral strain from the *Bunyaviridae* family revealed from the DNA Sequencing analysis of the samples.

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APPENDICES

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

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

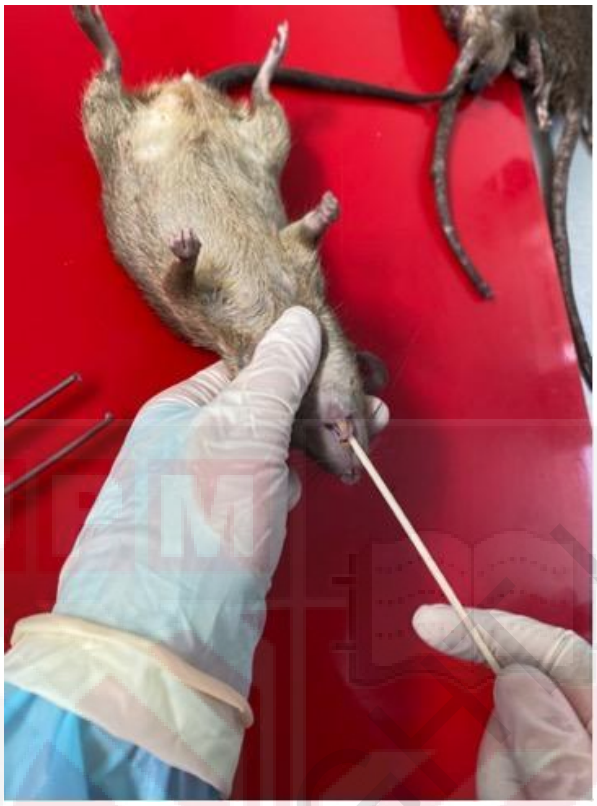
Appendix 3. Baits that were prepared to attract the rats into the cage trap.

Appendix 4. Placement of traps around the market areas.

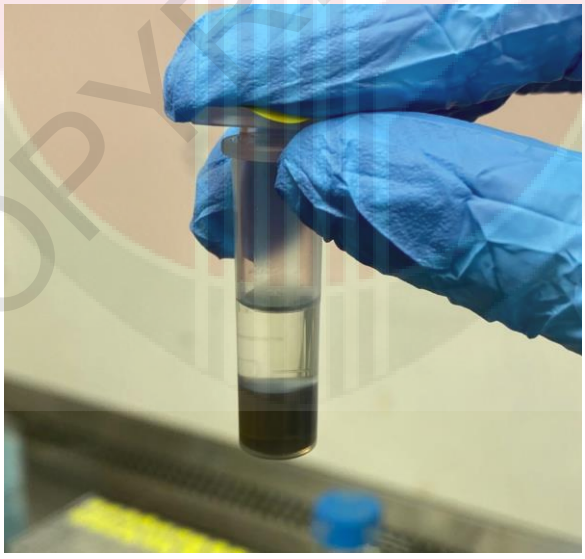
Appendix 5. Anaesthesia of rats using chloroform was done by trapping in a container box.

Appendix 6. Collection of blood via intracardiac puncture.

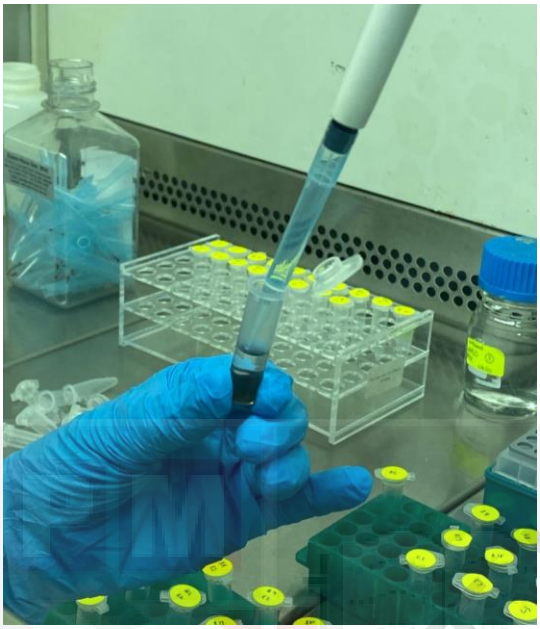
Appendix 7. Collection of oropharyngeal swab using sterile cotton swab.



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.