DETECTION OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 (SARS-CoV-2) FROM OROPHARYNGEAL SWAB SAMPLE OF CATS IN KELANTAN BY USING REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) METHODS

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A RESEARCH PAPER SUBMITTED TO THE FACULTY OF VETERINARY MEDICINE, UNIVERSITI MALAYSIA KELANTAN
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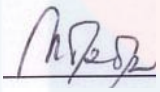
CERTIFICATION

This is to certify that we have read this research paper entitled "**Detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) From Oropharyngeal Swab Sample of Cats in Kelantan by Using Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Methods"** by Nik Nur Aidawatie Binti Nik Mat, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course DVT 55204 – Research Project.



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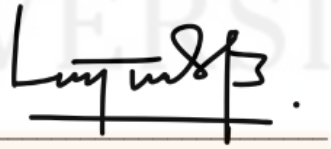

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Thank You

DEDICATIONS

I dedicate the results of my work to my family, especially my mother, Ramlah Ibrahim who supported me a lot and also my late father, Allahyarham Nik Mat who always supported me during his life. Million thanks to my small family for giving words of encouragement and always being by my side at the lowest moment throughout completing this work.

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LIST OF SYMBOLS AND ABBREVIATIONS

Bp	Base pair
nAb	Neutralizing antibody
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
RT-LAMP	Reverse Transcription Loop-mediated isothermal amplification
RT-PCR	Reverse Transcription Polymerase Chain Reaction
℃	Celsius
DNA	Deoxyribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
µL	microliter
F3	Forward outer primer
R3	Reverse outer primer
LF	Forward loop primer
LB	Reverse loop primer
FIP	Forward inner primer
BIP	Backward inner primer
F2	Forward primer
R	Reverse primer

ABSTRACT

Abstracts from research papers submitted to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan to fulfil part of the requirements of the course DVT 55204 – Research Project.

Coronavirus disease (COVID-19) can be defined as the new disease that caused by a virus which is a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that originally identified in the Wuhan City, Hubei Province, China (Cennimo, 2022). Apart from humans being infected and spreading Covid-19, there are studies stating that the disease can also infect vertebrate animals such as cats. To date, there is no study has been conducted on the presence of SARS-CoV-2 in cats from Kelantan, Malaysia. This research was conducted which aimed to detect the presence of SARS-CoV-2 in cats from Kelantan. Sixteen swab samples from the oropharyngeal of cats were obtained from the archived samples of the virology laboratory of Faculty of Veterinary Medicine (FPV), UMK. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) method used to detect the presence of SARS-CoV-2 and further confirmed with reverse transcription polymerase chain reaction (RT-PCR) as a gold standard molecular method. One of the samples was positive with the RT-LAMP method but not with RT-PCR. This result indicates that SARS-CoV-2 presence in cats from Kelantan and RT-LAMP is more sensitive than RT-PCR. However, virus isolation needs to be conducted to further confirm this result.

Keywords: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Kelantan's cats, RT-LAMP, RT-PCR

ABSTRAK

Abstrak daripada kertas penyelidikan yang diserahkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan.

Penyakit Coronavirus (COVID-19) boleh ditakrifkan sebagai penyakit baharu yang disebabkan oleh virus yang merupakan novel sindrom pernafasan akut teruk coronavirus 2 (SARS-CoV-2) yang pada asalnya dikenal pasti di Bandar Wuhan, Wilayah Hubei, China (Cennimo, 2022). Selain manusia dijangkiti dan menyebarkan Covid-19, terdapat kajian menyatakan penyakit itu juga boleh menjangkiti haiwan vertebrata seperti kucing. Sehingga kini, tiada kajian dijalankan mengenai kehadiran SARS-CoV-2 dalam kucing dari Kelantan, Malaysia. Penyelidikan ini dijalankan bertujuan untuk mengesan kehadiran SARS-CoV-2 dalam kucing dari Kelantan. Enam belas sampel swab daripada oropharyngeal kucing diperoleh daripada sampel arkib makmal virologi Fakulti Perubatan Veterinar (FPV), UMK. Kaedah penguatan isoterma pengantara gelung transkripsi terbalik (RT-LAMP) digunakan untuk mengesan kehadiran SARS-CoV-2 dan seterusnya disahkan dengan tindak balas rantai polimerase transkripsi terbalik (RT-PCR) sebagai kaedah molekul standard emas. Salah satu sampel adalah positif dengan kaedah RT-LAMP tetapi tidak dengan RT-PCR. Keputusan ini menunjukkan bahawa kehadiran SARS-CoV-2 dalam kucing dari Kelantan dan RT-LAMP adalah lebih sensitif daripada RT-PCR. Walau bagaimanapun, pengasingan virus perlu dijalankan untuk mengesahkan lagi keputusan ini.

Kata kunci: Sindrom pernafasan akut yang teruk coronavirus 2 (SARS-CoV-2), Kucing Kelantan, RT-LAMP, RT-PCR

1.0 INTRODUCTION

Covid-19 which is hitting the whole world starting in 2019 is giving a great blow to the whole of humanity as this epidemic is easily spread and has a very bad effect on health. Based on the Covid Now government website, 35 680 deaths have been recorded during the 3 years Covid-19 hit Malaysia (World, 2020). COVID-19 can be defined as the new disease that caused by a virus which is a novel Coronavirus which called as a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that originally identified in the Wuhan City, Hubei Province, China (Cennimo, 2022).

The non-segmented, 30-kb positive-sense RNA genome of coronaviruses is non-transcribed. The genome functions as an mRNA for the translation of the replicase polyproteins because it has a 5′ cap structure and a 3′ poly (A) tail (Fehr & Perlman, 2015). Apart from humans being infected and spreading Covid-19, there are studies stating that the disease can also affect vertebrate animals such as cats. To confirm the statement, there are few laboratory methods that can be done to detect the presence of the virus such as reverse transcription loop-mediated isothermal amplification (RT-LAMP) and reverse transcription (RT-PCR) that will be discussed in this study.

Thus, reverse transcription loop-mediated isothermal amplification (RT-LAMP) and reverse transcription polymerase chain reaction (RT-PCR) method was carried out in this study to detect the presence of SARS-CoV-2 in cats from Kelantan, Malaysia.

1.1 Research problem

Coronavirus disease-2019 (COVID-19) is a respiratory disease that affects the lung. Worldwide reports of SARS-CoV-2-infected animals are available. Most of these animals contracted COVID-19 after coming into touch with owners, caregivers, or other persons in close proximity. The whole list of animals that can contract the disease is still unknown (CDC, 2020). To confirm this statement provided, hence it is important to confirm that SARS-CoV-2 that affects humans can transmit the Covid-19 disease to cat which is important for future control of the infection.

1.2 Research questions

1.2.1 Is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) present in cats from Kelantan.

1.2.2 Is RT-LAMP more sensitive than RT-PCR.

1.3 Research hypothesis

1.3.1 Coronavirus 2 (SARS-CoV-2) is present in cats from Kelantan.

1.4 Objectives

To determine the presence of SARS-CoV-2 in cats from Kelantan, Malaysia using RT-LAMP and RT-PCR methods.

2.0 LITERATURE REVIEW

2.1 General characteristic of Coronavirus 2 (SARS-CoV-2)

Coronaviruses (CoVs) are enveloped single-stranded positive sense RNA viruses that require real time polymerase chain (RT-PCR) for detection. The virus also has a spike-shaped glycoprotein membrane. Covid-19 is a dangerous pandemic that is easily spread. COVID-19 is a respiratory disease that is mostly transmitted by airborne droplets (Hashim et al., 2021). Coronavirus can cause fever in humans and certain illness or asymptomatic in the animals such as cattle, bats and camels (CDC, 2020).

Figure 1 shows the morphological of the coronavirus including SARS-CoV-2. It contains envelope protein (E), membrane glycoprotein (M), Nucleocapsid protein (N)and spike glycoprotein that play important role in penetrating host cells and causing the infection (Duan et al., 2020).

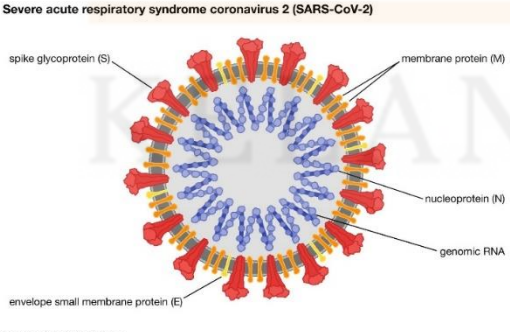

Figure 1: Coronavirus structure

Source: Encyclopedia Britannica

2.2 Presence of acute respiratory disease syndrome coronavirus 2 (SARS-CoV-2) in animals

Animals also are not excluded from being infected with SARS-Cov-2. The causative agent of the coronavirus disease 2019 (Covid-19) able to infect few types of domestic animals including captive and wildlife. There is some data collected by previous study that was conducted in North-Easter Spain that reveal presence of antibody against this virus in pets. Despite the low incidence of active SARS-CoV-2 infection, as determined by direct viral RNA detection (0.3%), COVID-19-positive households had a rather high prevalence of nAbs in their pets (near to 25% in cats, 10% in dogs, and 40% in ferrets) (Fernández-Bastit et al., 2022). Thus, it is important to monitor the pets that have frequent contact with human as the companion animals can be infected by SARS-CoV-2 too.

2.3 Nucleic acid method

2.3.1 Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Method

The potent new gene amplification method known as "loop mediated isothermal amplification" (LAMP) is becoming a popular quick diagnostic tool for the early detection and identification of pathogens. The entire process is quick and easy; using a set of six specifically designed primers (F3, FIP, LF-Forward primers and B3,BIP and LB-Reverse primers) that cover eight different regions of a target gene under isothermal conditions, the amplification may be finished in less than one hour by incubating all the reagents in a single tube (Wong et al., 2018). It can be done in the heating block with temperature set to 60℃.

Real-time RT-PCR (qRT-PCR) is a gold standard laboratory test for the detection of Covid-19 infection (Chaimayo et al., 2020). An alternative to this qRT-PCR is reverse transcriptase loop-mediated isothermal amplification (RT-LAMP). It is utilised for the amplification of RNA templates with significant strand displacement activity. The method is fast since it uses only one amplification temperature (60-65^{o}C) for one to two hours incubation and requires less resources (El-Kafrawy et al., 2022). LAMP response was denoted by the colour green, whereas negative LAMP reaction was denoted by the colour orange (Wong et al., 2018)

As an RNA virus, SARS-CoV-2 is one of the viruses that can be detected through RT-LAMP method. The most popular technique for detecting COVID-19 includes the use of pH-based colorimetric tests with findings that can be seen with the unaided eye.

2.3.2 Reverse Transcription - Polymerase Chain Reaction.

RT-PCR is one of the valuable method for the detection of RNA. Reverse transcriptase transforms RNA molecules into their complementary DNA (cDNA) sequences in a process known as reverse transcription (RT) process (Farrell, 2010) . Next, the freshly generated cDNA is amplified using conventional PCR techniques. It is a two-step process and required around three hours to finish. However, to speed-up the reaction, RT-PCR can also be conducted using commercial one-step RT-PCR system kits. The successful rate is quite high. Promoting an appropriate balance between template specificity, thermodynamic stability when base-paired to the template, and the ability of one primer to work with the other(s) to promote RT-PCR is necessary for designing effective primers (Farrell, 2010). The determination of the result can be interpreted by running the product into gel electrophoresis and required UV light using UV transluminator to able to see the band of the specific base pair.

3.0 MATERIALS AND METHODS

3.1 Information on samples

Sixteen swab samples from the oropharyngeal of cats were obtained from the archived samples of the virology laboratory of Faculty of Veterinary Medicine (FPV), UMK. Sample was taken from previous study that had been conducted by final year student of 2021. The swab samples were taken from oropharyngeal of 16 cats in Kelantan area. The used of the animals for the previous study was being approved ethically as it follows the guidelines. A total 16 cats selected in Kota Bharu, Kelantan and the procedures performed on the cats were done under supervision of veterinarian who ensured that students were done the procedure ethically.

3.1 RNA Extraction from oropharyngeal swab samples

The oropharyngeal samples were extracted using GENEzol reagent (Geneaid Biotech Ltd, Taiwan) which contain phenol, chloroform and guanidine isothiocyanate. The RNA extraction protocol was done following the manufacturer's protocol. The extraction was started by adding 750 μL of GENEzol reagent into a 250 μL homogenate sample and incubated at room temperature for 5 minutes. A 200 μL chloroform was added and was shaken vigorously for 15 seconds using a vortex (Corning, Japan). Then, all the tubes containing samples were inserted into a centrifuge and centrifuged at 14 000g for 15 minutes at 4℃ and the precipitation of RNA could be observed at the bottom of the tubes as white or translucent pellet. The aqueous phase (supernatant) with amount 600 μL was transferred into the new eppendorf tube by using micropipette. Isopropanol solution with an amount of 600 μL was added into the pellet inside the tube using a micropipette and kept at room temperature for 10 minutes. It was then continued to be centrifuged at 14 000g for 10 minutes at 4℃. The RNA precipitate forms a white pellet on the bottom of the tubes. The supernatant was removed, and the pellet was air-dried for 10 minutes. Thirty (30) μL of nuclease free water was added into the pellet in each of the tubes and a gentle mix was done by pipetting up and down. The tubes were incubated at 55℃ for 10-15 minutes and were stored in -80℃ until further used for RT-PCR.

3.2 RT-LAMP

The reaction was carried out in a 25 μL reaction mixture using IsoFast *Bst* Mix reagent kit (PCR Biosystems, UK). Master mix was prepared as in Table 1, 10x primer set 2.50 μL. The preparation was set up on ice. The mixture was vortexed to homogenise them. Then, 5 μL of RNA extraction was added into the mixture prepared in the (Table 1). Primers used (Table 2) were designed based on RNA dependent RNA polymerase (RdRP) SARS-CoV-2 gene sequences obtained from the National Center for Biotechnology (NCBI) GenBank Information . Positive and negative control were also included. The samples were heated at 63 ℃ for 1 hour 30 minutes. The products were visualised directly using naked eyes by observing the colour change from orange to fluorescent green.

Table 1: RT-LAMP mixture, volume and concentration of the reagent.

Component	Volume
2x IsoFast Bst Mix	12.50 µL
20x Fluorescent Dye	1.25 µL
RTase Go (RT+ *Bst* polymerase enzymes)	2.00 µL
Complete Forward primers (5 ρmol F3, 40 ρmol FIP and 20 ρmol LF)	1 µL
Complete Reverse primers (5 ρmol B3, 40 ρmol BIP and 20 ρmol LB)	1 µL 1 µL
RNase Inhibitor	0.3 µL
Nuclease-free water	1.95 µL
Total volume	**25 µL**

Table 2: RT-LAMP Primers

Primer	**Primer Sequence (5'-3')**	**Source**
F3	**5'- GAA ATG GTC ATG TGT GGC GG – 3'**	

R3	**5'- GAG ACA CTC ATA AAG TCT GTG – 3'**	**This study**
LF	**5'- GCA TCT CCT GAT GAT GTT C – 3'**	
LB	**5'- GCA CTT TTA TCT ACT GAT GG – 3'**	
FIP	**5'- GTT AAA AAC ACT ATT AGC ATT TTT TCA CTA TAT GTT AAA CCA GG – 3'**	
BIP	**5'- GCT GTC ACG GCC AAT GTT ATT TTT TGC GGA CAT ACT TAT CGG C – 3'**	

3.3 RT-PCR

The reaction was carried out in a 25 µL reaction mixture using AccessQuick RT-PCR reagent kit (Promega, USA) from of the extracted RNA products from oropharynx swab sample. For RT-PCR reaction, five µL of the extracted RNA product from each sample is preheated at 95°C for 5 minutes and immediately placed on the ice. Each of the extracted RNA from the sample will be added into 20 µL of reaction mixture containing 12.5 µL of 2 x AccessQuick buffer, 0.5 µL of AMV reverse transcriptase enzyme, 1µL of forward and reverse primer with 20 pmol concentration, 0.5 µL (8U) Taq polymerase, 4.3 µL of nuclease free water following the protocol recommended by the manufacturer (Promega, USA) with slight modification. RT-PCR reaction was performed using thermocycler (Biorad T100, USA). The primers used as listed in the (Table 3) that were designed based on SARS-CoV-2 RdRP gene sequences obtained

from the NCBI GenBank as while the reaction cycle for one-step RT-PCR is shown in (Table 4).

Table 3: RT-PCR Primer Sequences, Volume and Concentration

Primer	Sequence	Volume and Concentration	Product size (bp)	Reference
RdRP F	**5'- GTG ARA TGG TCA TGT GTG GCG G -3'**	**1 µL, 20 pmol**	**220 bp**	**This study**
RdRP R	**5' – CAR ATG TTA AAS ACA CTA TTA GCA TA – 3'**	**1 µL, 20 pmol**		

Table 4 : RT-PCR Reaction, Temperature ℃ , Duration and Number of Cycles

Reaction	Temperature ℃	Duration (min)	Number of cycles
Pre-heat RNA	90	5	-
cDNA Synthesis	45	60	1
RT inactivation	65	5	1
Denaturation	90	0.5	35

Annealing	50	0.5	35
Extension	72	0.5	35
Post- extension	72	7	1
Hold	15	∞	

3.4 Gel Electrophoresis

Preparation of 1.5% gel was done prior to separating the DNA by length and size as well as for visualization. Approximately around 1.2g of agarose powder and 80ml of 1 x Tris-borate-EDTA (TBE) buffer was mixed inside the glass media bottle and microwaved for 2 minutes until fully dissolved. Midori Green DNA stain (NIPPON Genetics, Japan) at an amount of one µL was added to the warmed agarose gel and swirled was applied to mix the solution. The stain functions by intercalating DNA and it will emit green fluorescence to make the gel loaded with DNA to be visible under UV light. The gel solution was poured into the gel casting tray with 10 wells-comb and was allowed to solidified in 30 minutes. The solidified agarose gel was loaded into the gel box that was filled with 1 x TBE buffer. The buffer level must be enough to cover the gel before loading the sample into the well. DNA ladder of five µLwas loaded into the first lane of the well. A five µL of each RT-PCR product was mixed with one µL of 6X loading dye and pipetted into well. Next, the gel was run at 100V for 40 minutes from negative to positive charge until the dye covered 80% of the way down. The gel was visualized with Gel Doc Imager (Bio-Rad, USA)

4.0 RESULTS

4.1 RT-LAMP Result

Out of 16 oropharyngeal cat samples, one sample (S16) was positive with fluorescent light green colour including the positive sample (Figure 2).

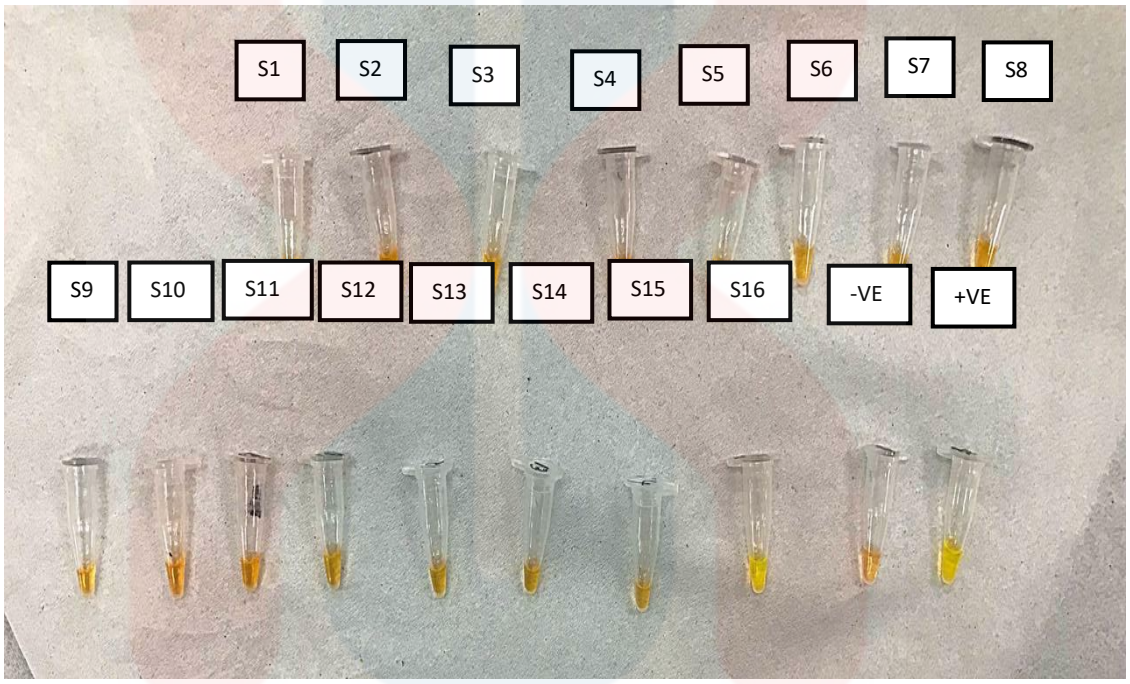

Figure 2: Detection of RT-LAMP product using fluorescent dye by direct observation method. Sample Sample 16 (S16) shows fluorescent light green indicating that it is contain Coranavirus viral nucleic acid while orange colour (Sample 1,2,4,5,6,7,8,9,10,11,12,13,14,15) indicate negative for Coronavirus.

4.2 RT-PCR result

Based on the RT-PCR accompanied by 1.5% gel electrophoresis, all oropharyngeal swab sample of the cats were negative for SARS-CoV-2 as shows in the table 5 and only positive control shows the expected band of 220 base pairs (bp) (Figure 3), indicating the presence of SARS-CoV-2 RNA in the positive control.

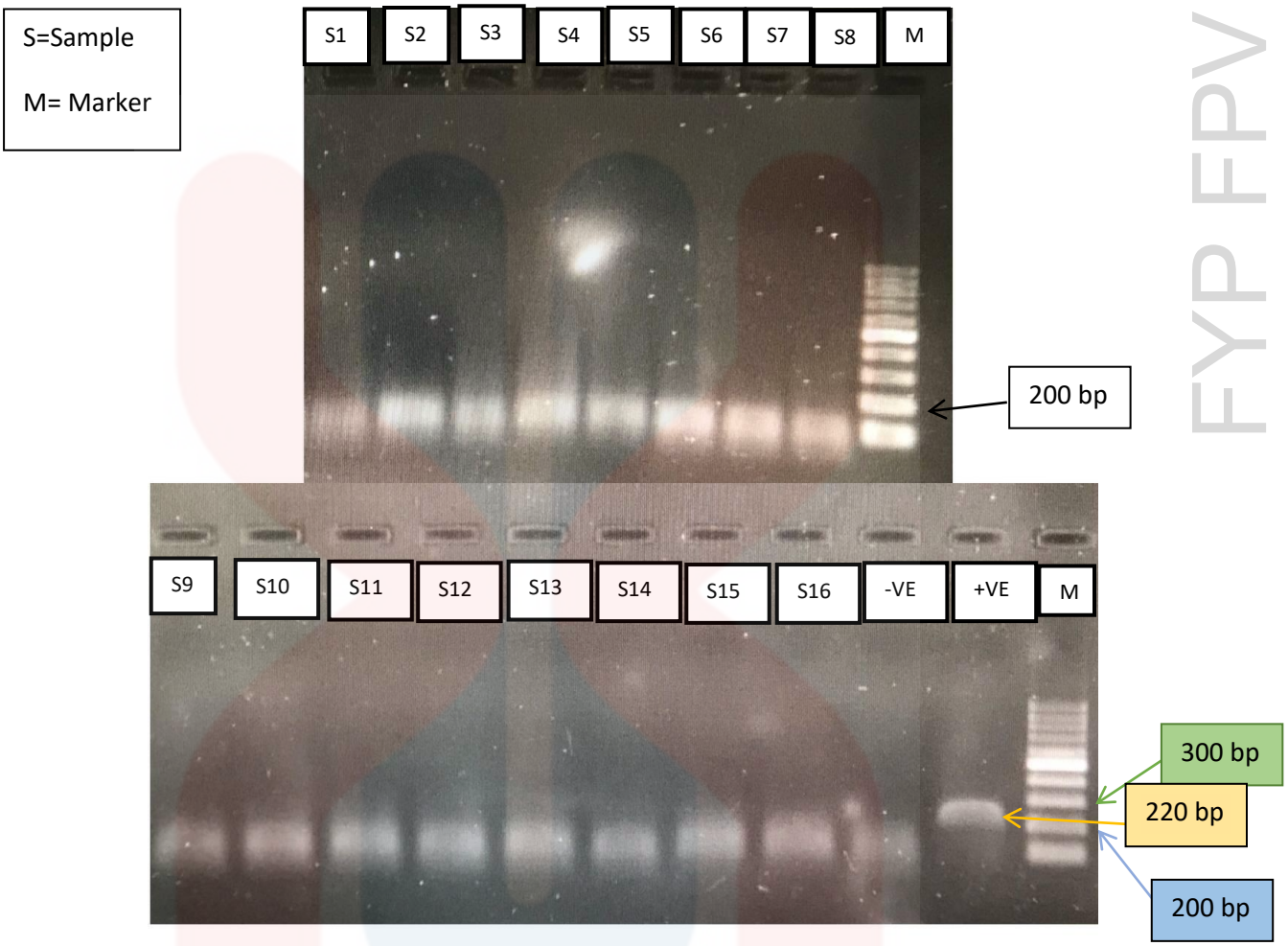

Figure 3: RT-PCR from oropharynx swab samples of cats by using RNA extraction kit. All shows negative results and only positive control at 220 bp can be observed.

Sample	Result
S1	Negative
S2	Negative
S3	Negative
S4	Negative
S5	Negative
S6	Negative
S7	Negative
S8	Negative
S9	Negative
S10	Negative
S11	Negative
S12	Negative
S13	Negative
S14	Negative
S15	Negative
S16	Negative

Table 5 : RT-PCR Result

5.0 DISCUSSION

SARS-CoV-2 can easily spread through air droplets and early detection and prevention is needed. Most animals contracted COVID-19 after contacting with their owners, caregivers, or other persons in close proximity. (CDC,2020). However, animals that are affected may and not may exhibit clinical signs. In this study we have detected one cat sample (S16) positive for SARS-CoV-2 using RT-LAMP method. It is an expected result since the sample (S16) was obtained from positive-Covid-19 owners and there was a possibility that the cat had the infection after contact with its owner as previously reported.

In this study, positive result was only obtained by RT-LAMP and negative by conventional RT-PCR as LAMP method was shown previously to be more sensitive than PCR. Besides that, this RT-LAMP assay used a one-tube assay system and incubated using a simple heating block and the result was visualised directly by naked eyes without the need of thermocycler and agarose gel electrophoresis as in RT-PCR (Amaral et al., 2021). However, virus isolation needs to be conducted to further confirm this finding. Considering this simplicity, rapidness, and sensitivity, RT-LAMP has the advantage of the detection of SARS-CoV-2 at low-resourced settings, in the poorly-equipped laboratory compared to RT-PCR that needs sophisticated equipment such as a thermocycler (Moore et al., 2021). This RT-LAMP assay, however, needs to be optimised for their sensitivity and specificity for future use on the field samples.

6.0 CONCLUSION AND RECOMMENDATION

In conclusion, SARS-CoV-2 is present in cat from Kelantan as detected by RT-LAMP method. RT-LAMP is more simple, sensitive and rapid than conventional RT-PCR and can be recommended for the detection of SARS-CoV-2 in human or animals at low-resourced settings in the future.

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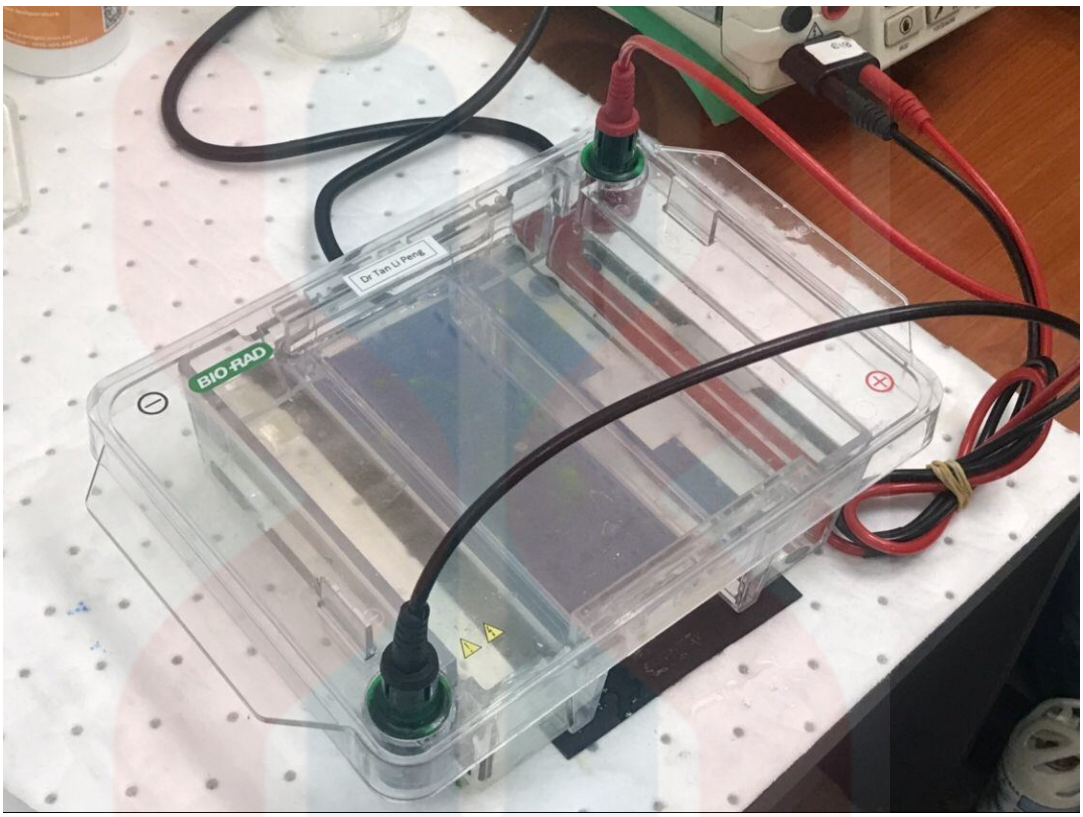
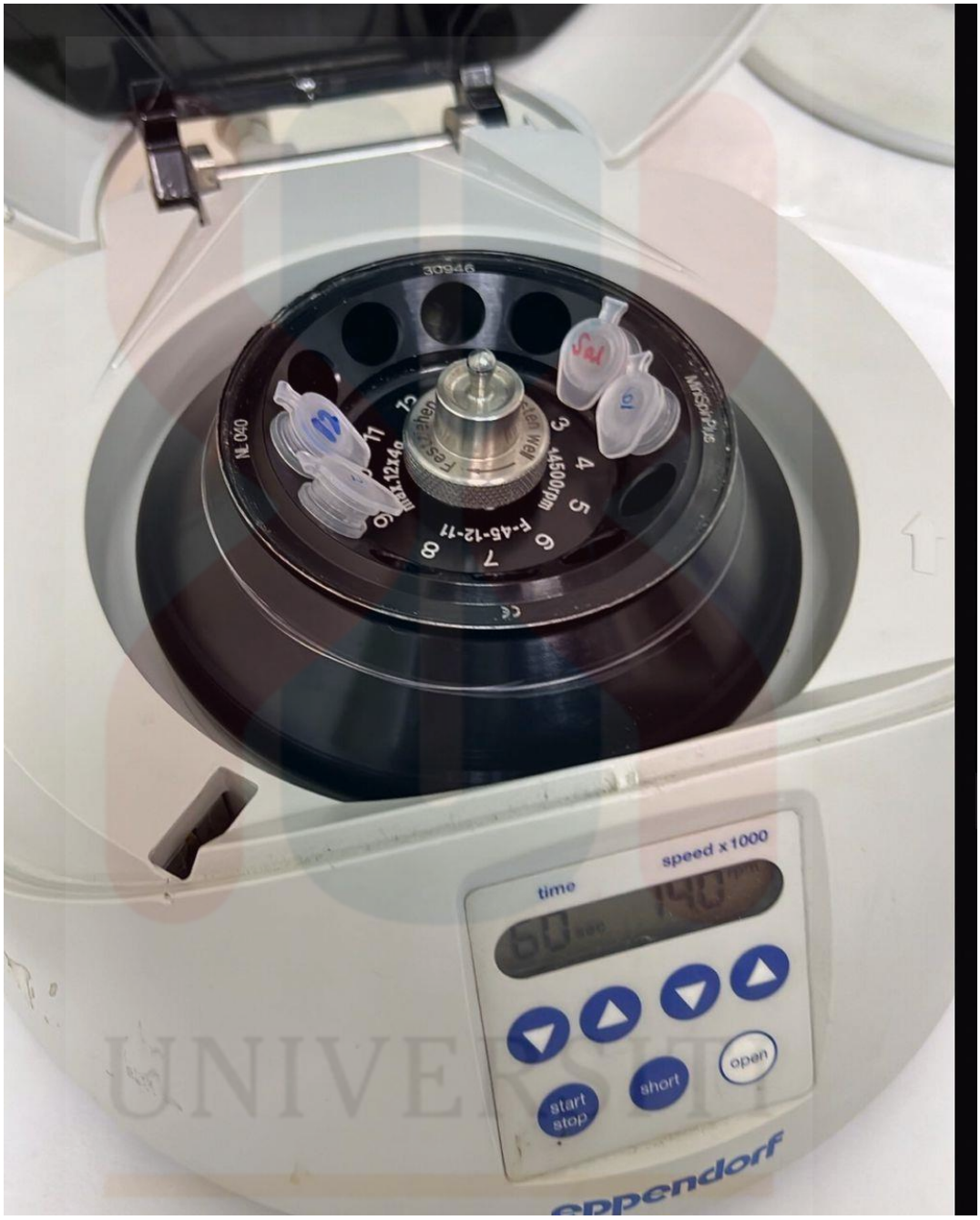
Appendix

Appendix A: RT-LAMP incubation of extracted oropharynx swab samples from cats using heating block at 63℃



Appendix B: Gel electrophoresis of RT-PCR product for 40 minutes



Appendix C: Mini spin centrifuge to homogenized the sample and reagent in the microtubes