MOLECULAR DETECTION OF SARS-COV-2 (COVID-19) IN STRAY CATS IN KOTA BHARU, KELANTAN

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Molecular Detection of SARS-COV-2 in Stray Cats in Kota Bharu, Kelantan'** by Jeremy Tan Jianhao, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT55204 – Research Project.

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Thank You

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ABSTRACT

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

In late 2019, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) emerged in the city of Wuhan, China and quickly spread all over the world causing the latest pandemic. Due to the close association between humans and animals including companion animals, livestock and wildlife species, the potential transmission of SARS-CoV-2 from humans to animals, which is known as "reverse zoonosis" as well as the potential role infected animals might have in the spread of the disease has become a concern. This study was done to detect the presence of SARS-CoV-2 infection in stray cats in Kota Bharu, Kelantan. Oropharyngeal swabs were taken from 15 stray cats from Kota Bharu, Kelantan where the RNA was extracted, amplified with reverse transcription-polymerase chain reaction (RT-PCR) and run through agarose gel electrophoresis. The results revealed that all cats tested negative for SARS-CoV-2 infection thus, concluding that there is a 0% occurrence of SARS-CoV-2 infection in stray cats from Kota Bharu, Kelantan.

Keywords: SARS-CoV-2, COVID-19, RT-PCR, stray cats, reverse zoonosis, occurrence

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT55204 – Research Project.

Pada penghujung 2019, Sindrom Pernafasan Akut Teruk Coronavirus 2 (SARS-CoV-2) muncul di bandar Wuhan, China dan merebak dengan cepat ke seluruh dunia menyebabkan wabak terkini. Disebabkan perkaitan rapat antara manusia dan haiwan termasuk haiwan peliharaan, ternakan dan spesies hidupan liar, potensi penularan SARS-CoV-2 daripada manusia kepada haiwan, yang dikenali sebagai "zoonosis terbalik" serta potensi haiwan untuk memainkan peranan dalam penyebaran penyakit ini telah menjadi kebimbangan. Kajian ini dilakukan untuk mengesan jangkitan SARS-CoV-2 pada kucing liar di Kota Bharu, Kelantan. Swab oropharyngeal telah diambil daripada 15 kucing liar dari Kota Bharu, Kelantan di mana RNA diekstrak, digandakan dengan ‘reverse transcription-polymerase chain reaction’ dan diproses melalui elektroforesis gel agarose. Keputusan menunjukkan semua kucing yang diuji adalah negatif untuk jangkitan SARS-CoV-2 dan kehadiran jangkitan SARS-CoV-2 dalam kucing liar dari Kota Bharu, Kelantan adalah 0%.

Kata kunci: SARS-CoV-2, COVID-19, RT-PCR, kucing liar, zoonosis terbalik, kehadiran

1.0 INTRODUCTION

Coronaviruses, found under the family *Coronaviridae*, have a positive-strand RNA genome and an envelope (Van der Hoek et al., 2004). They are spherical in shape with 125nm diameter in size and covered with club-shaped spikes on the surface which gives them a solar corona appearance (Malik, 2020). Coronaviruses are vastly diverse and are able to infect various animals including humans and cause respiratory infections that range for mild to severe cases (Hu et al., 2020). In late 2019, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) began in the city of Wuhan, China and quickly spread throughout the world causing the latest pandemic (Hu et al., 2020). SARS-CoV-2 appears to have also originated from bats as seen in SARS-CoV and MERS-CoV (Malik, 2020).

As animals have close associations with humans as pets, livestock and even wildlife species, the potential for SARS-CoV-2 to transmit from humans to animals, which is known as "reverse zoonosis" as well as the possible role any animals that are infected might have in the spread of the disease has become a concern (Gaudreault et al., 2020). Currently, no evidence has been found to indicate that animals have a significant role in the transmission of SARS-CoV-2 to humans (Centre for Disease Control and Prevention, 2022). However, there have been documentation of suspected human-to-mink transmission in a mink farm in USA, Italy, Spain, France, Greece, Poland, Denmark, Lithuania and Netherlands (Hosie et al., 2021). Additionally, there have also been many reports where domestic animals cohabiting with owners who had been positively tested for SARS-CoV-2 and this infection is presumed to be from their owners (Hosie et al., 2021). In cats, SARS-CoV-2 infections can show various severity from asymptomatic cases as seen in the first positive tested cat in Hong Kong to cough and shallow breathing as seen in the first positive tested cat in Europe (Hosie et al., 2021).

This research is conducted because Malaysia is a country that possesses a large population of stray cats and dogs with Kelantan being populated with more cats. It is a significant concern of the general public whether these animals are infected with SARS-CoV-2 and can further spread the virus to other humans in the population.

Thus, this research is done to find evidence of SARS-CoV-2 infection in stray cats in Kota Bharu, Kelantan.

1.1 Research Problem

In Malaysia, the first positive case of COVID-19 was detected on the 25th of January 2020 and the virus has since spread throughout the country. Based on several reports, there is small but possible chance of transmission of SARS-CoV-2 from humans to animals. Despite the many reported COVID-19 cases in humans in Kelantan, there are no published data on studies of the disease in stray cats of Kelantan. Thus, this research aims to detect the presence of COVID-19 in stray cats in selected areas in Kelantan using reverse transcription-polymerase chain reaction.

1.2 Research Questions

1.2.1. Is SARS-COV-2 infection present in stray cats in Kota Bharu, Kelantan?

1.3 Research Hypothesis

1.3.1. SARS-COV-2 infection is present in stray cats in Kota Bharu, Kelantan.

1.4 Research Objectives

1.4.1. To detect the presence of SARS-COV-2 infection in stray cats in Kota Bharu, Kelantan by using reverse transcription-polymerase chain reaction.

2.0 LITERATURE REVIEW

2.1 Characteristics of SARS-CoV-2

SARS-CoV-2 is a positive-sense single-stranded RNA virus with an envelope and a non-segmented genome about 30kb in size. (Uddin et al., 2020). Covering the envelop are membrane (M) proteins and envelope (E) proteins that are found among the spike (S) proteins (Yang et al., 2020). Within the envelope, a spiral nucleocapsid is formed from the genomic RNA and phosphorylated nucleocapsid (N) protein (Yang et al., 2020). There are four genera of coronaviruses, α, β, γ, and δ based on genetic and antigenic characteristics where mammals are infected by only α and β coronaviruses, while birds are mainly infected γ and δ (Yang et al., 2020).

2.2 Clinical Features of SARS-CoV-2

Clinical manifestations differ with age with the disease being more severe the older the individual infected (Hu et al., 2020). The majority of young individuals and children infected are either asymptomatic or show mild signs while a higher risk of developing severe respiratory disease that requires hospitalization occurs in older men above the age of 60 years with co-morbidities (Hu et al., 2020). The most commonly seen symptoms upon infection are fever, dry cough and fatigue while sore throat, sputum production, chest pain, headache, haemoptysis, chill as well as gastrointestinal signs such as diarrhoea, anorexia, nausea and vomiting are less commonly seen (Hu et al., 2020). Most signs of COVID-19 begin after a 1-14 days incubation period (Hu et al., 2020). According to studies done by Abdel-Moneim and Abdelwhab, cases of SARS-CoV-2 infection in animals, including cats, are asymptomatic, show mild respiratory clinical signs or gastrointestinal clinical signs (Adbel-Moneim & Abdelwhab, 2020).

2.3 Studies of SAR-CoV-2 Infection in Animals

A surveillance was done in Hong Kong on 27 dogs where two dogs tested positive but were both asymptomatic (Adbel-Moneim & Abdelwhab, 2020). However, 13 dogs from France and Northern Spain that were housed with infected individuals

tested negative which suggests that dogs does not have a major role in the transmission of COVID-19 (Adbel-Moneim & Abdelwhab, 2020).

In cats, various reports as mentioned by Adbel-Moneim and Abdelwhab as well as Hosie et al. have suggested human-to-cat transmission as cases in China, Hong Kong, Belgium, USA and Northern Spain all show cats that have tested positive after being kept in the same household as individuals infected with the SARS-CoV-2 (Adbel-Moneim & Abdelwhab, 2020; Hosie et al., 2021). It is unclear if cats can play a role in human or animal transmission of the virus, but findings reveal that pet cats may be more susceptible to SARS-CoV-2 as compared to dogs (Adbel-Moneim & Abdelwhab, 2020).

In wildlife, lions and tigers from the Bronx Zoo in New York City have also tested positive in April 2020 and showed respiratory signs such as cough and also inappetence (Adbel-Moneim & Abdelwhab, 2020). A test on other co-housed animals was negative which suggest poor animal-to-animal transmission and also suggests that it was most likely the asymptomatic infected zookeeper that transmitted the virus to them. (Adbel-Moneim & Abdelwhab, 2020).

2.4 The Use of Reverse Transcriptase Polymerase Chain Reaction to Detect SARS-CoV-2

Currently, cats are recommended to be tested for SARS-CoV-2 in laboratories that have level 3 containment facilities as handling the virus puts the laboratory staff at risk as well (Hosie et al., 2021). Swab samples taken from the oropharynx, nasal and oral cavities can be used to test for the presence of SARS-CoV-2 by using RT-PCR (Hosie et al., 2021). In cases where gastrointestinal clinical signs such as vomiting and diarrhoea are seen, feces and vomitus can also be sent for testing (Hosie et al., 2021). However, swabs from the respiratory tract have been found to be more consistent to test positive.

3.0 METHODOLOGY

This study was approved by the Institutional Animal Care and Use Committee Universtiti Malaysia Kelantan to involved the use and care of animals with the ethics code: UMK/FPV/ACUE/FYP/024/2022.

3.1 Sample Collection

A total of 15 oropharyngeal swab samples were obtained from 15 stray cats selected in Kota Bharu, Kelantan. All procedures performed on the cats were done under the supervision of a senior veterinarian who ensured that they were done properly and ethically. Individuals involved in handling the cats were given personal protective equipment including a facemask, gloves, and a laboratory coat to ensure their safety and protection.

Cats were selected, captured, and brought to the animal holding room in the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The cats' mouth were held open by a restrainer while the oropharyngeal swab was collected from the oropharyngeal area. The swab was carefully retrieved to prevent any contamination. It was then stored in a 1.5ml screw cap tube prefilled with virus shield solution to ensure nucleic acid stability during sample collection and inactivates nucleases and infectious agents. The tubes were then stored in an icebox to be transported back to the laboratory where they were immediately stored at -80°C.

3.2 RNA Extraction

QIAamp Viral RNA Mini Kit (250) (Qiagen, USA) containing 250 QIAamp Mini Spin Columns, carrier RNA, collection tubes (2ml) and RNase-free buffer was used to extract and purify the 15 samples collected in a biosafety level 3 containment laboratory in the Faculty of Veterinary Medicine of University Malaysia Kelantan. The positive control used in this research was acquired from Hospital Raja Perempuan Zainab II, Kelantan.

Five hundred and sixty (560) µl Buffer AVL containing carrier RNA was pipetted into a 1.5ml microcentrifuge tube. One hundred and forty (140) µl solution from the sample collected was added to the microcentrifuge with the Buffer AVL-

carrier RNA. The tube was then incubated at room temperature for 10 minutes followed by centrifugation to remove any drops from the inside of the lid. Five hundred and sixty (560) µl ethanol (96%-100%) was added to the sample and a pulse vortex was used to mix the solution for 15 seconds.

Six hundred and thirty (630) µl of the solution was applied to the QIAamp Mini Spin Column (in 2ml collection tubes) without wetting the rim. The cap was closed and centrifugation at 6000 x g (8000 rpm) was done for 1 minute. Next, the QIAamp Mini Column was placed into a clean 2ml collection tube and the tube containing the filtrate was discarded and the process was then repeated. 500µl Buffer AW1 was added into the QIAamp Mini Column. The cap was closed and centrifugation at 6000 x g (8000 rpm) was done for 1 minute. The QIAamp Mini Column was then placed in a clean 2ml collection tube and the tube containing the filtrate was discarded. Next, 500µl Buffer AW2 was added into the QIAamp Mini Column. The cap was closed and centrifugation at full speed (20, 000 x g or 14, 000 rpm) was done for 3 minutes. The QIAamp Mini Column was placed in a new 2ml collection tube and the tube containing the filtrate was discarded. This was followed by centrifugation at full speed for 1 minute.

The QIAamp Mini Column was then placed in a clean 1.5ml microcentrifugation tube and 60µl Buffer AVE was added into the solution to bring it to room temperature. The cap was closed and incubation at room temperature was done for 1 minute. Next, centrifugation at 6000 x g (8000 rpm) was done for 1 minute. The extracted content was then stored at -80°C.

3.3 Detection of SAR-CoV-2 by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Agarose Gel Electrophoresis

The primers specific to detect SARS-Cov-2 were F3 (5'-GAAATGGTCATGTGTGG CGG-3') and R3 (5'-GAGACACTCATAAAGTCTGTG-3'). Using AccessQuick RT-PCR reagent kit (Promega, USA) for the reverse transcription-polymerase chain reaction, the master mix was prepared in a sterile 1.5ml microcentrifuge tube for all samples and prepared following the components of **Table 3.1**. Each run also included a positive control and negative control (Nuclease free water).

RT-PCR reactions were carried out with the following conditions: Initial denaturation at 95°C for 5 minutes, 35 cycles of 30 seconds denaturation at 70°C, 30 seconds annealing at 50°C, 30 seconds extension at 72°C and a final extension step of 72°C for 5 minutes.

Table 3.1: Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Components

Reagent	Volume per reaction (µL)	Volume for 15 reactions (µL)
2X access quick buffer	12.5	86
Nuclease free water	4.3	250
AMV-RT	0.5	10
Taq polymerase	0.5	10
RNAsin	0.2	4
Forward primer	1	20
Reverse primer	1	20

An agarose gel was prepared with 2% of 1.6g of agarose gel that was added to 80ml of TBE water that was microwaved for 3 minutes and addition of 1ml Midori green. One (1) ml of DNA markers and 4ml of the RT-PCR products were repeatedly pipetting up and down together to be mixed. The mixture is added into different chambers of the gel. The gel was then left to run for an hour at 100V. After an hour, the power was switched off and the gel was removed. A Gel Doc ™ EZ Imager (Bio-Rad, USA) was used to visualise the expected product size and was compared to the expected product size for SARS-CoV-2 which is 220 bp which was the positive control.

4.0 RESULTS

RT-PCR amplification of the extracted RNA from 15 oropharyngeal swabs using the protocol described above showed non-specific bands produced and seen in gel electrophoresis for sample 2, 4, and 5 seen in **Figure 4.1** and 6, 7, 8, 9, 10, 12, 13, 14, 15 seen in **Figure 4.2**. The positive controls seen in **Figure 4.1** were also faint but still visible. However, no positive results were seen in all 15 samples as compared to the positive control.

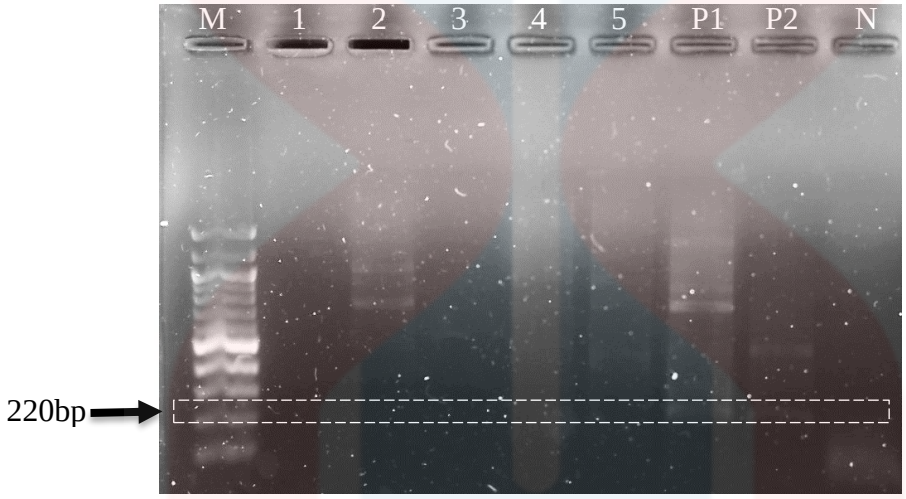

Figure 4.1: Agarose gel electrophoresis of RNA extracted from sample 1-5. M: Ladder marker; 1-5: isolated samples; P1, P2: Positive controls; N: Negative control.

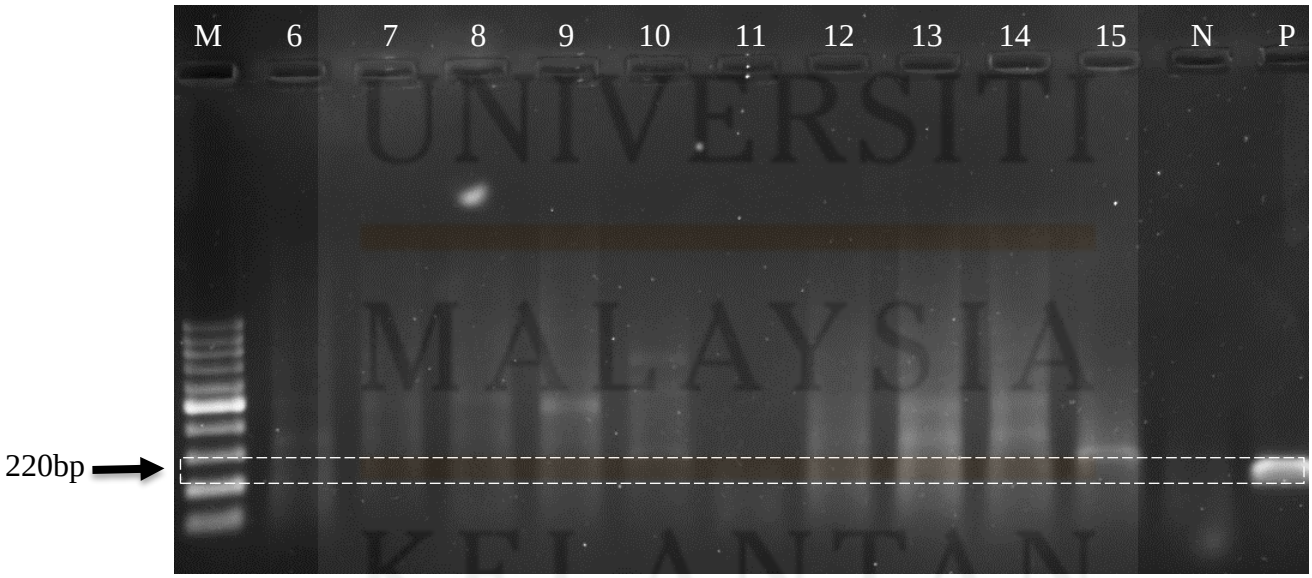

Figure 4.2: Agarose gel electrophoresis of RNA extracted from sample 6-15. M: Ladder marker; 6-15: isolated samples; N: Negative control; P: Positive controls.

Out of 15 cats sampled, only Cat 9 showed mild sneezing during the sampling process while all other cats did not show any respiratory symptoms as shown in **Table 4.1**.

Table 4.1: RT-PCR results compared to respiratory symptoms seen in the cats sampled

Cat	Respiratory Symptoms	Results from PCR
1	Negative	Negative
2	Negative	Negative
3	Negative	Negative
4	Negative	Negative
5	Negative	Negative
6	Negative	Negative
7	Negative	Negative
8	Negative	Negative
9	Positive	Negative
10	Negative	Negative
11	Negative	Negative
12	Negative	Negative
13	Negative	Negative
14	Negative	Negative
15	Negative	Negative

Thus, with the results from the RT-PCR conducted on the 15 oropharyngeal swabs collected from the stray cats, it is suggestive that the occurrence of SARS-CoV-2 in stray cats in Kota Bharu, Kelantan is at 0% as displayed by **Figure 4.3**.

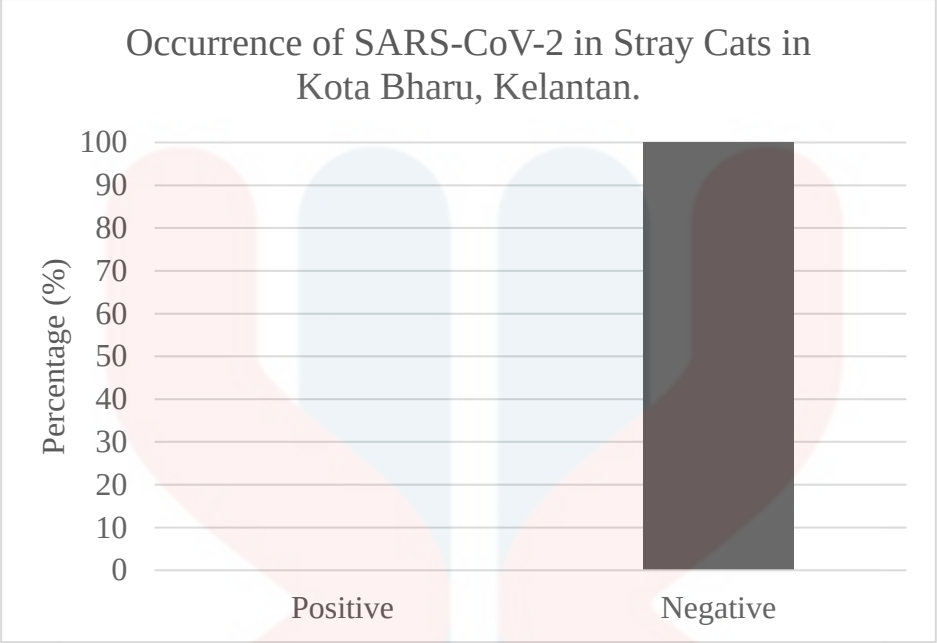

Figure 4.3: Occurrence of SARS-CoV-2 in stray cats in Kota Bharu, Kelantan.

5.0 DISCUSSION

All 15 stray cats sampled in this study tested negative which supports a previous study done on stray cats by Kuhlmeier et al. in 2022 where 882 stray cats sampled and tested by RT-qPCR were all negative for SARS-CoV-2 infections (Kuhlmeier et. al., 2022). Positive SARS-CoV-2 infection in animals have been seen to occur in cases where these animals have been kept in households where their owners are tested positive or when their keepers were tested positive (Abdel-Moneim & Abdelwhab, 2020; Hosie et al., 2021; Kannekens-Jager et al., 2022). In a recent study done by Kannekens-Jager et al., cats and dogs from a household with COVID-19 positive individual showed an 18.8% prevalence of SARS-CoV-2 infection as compared to the 1.8% observed in animals with an unknown history of exposure to a COVID-19 positive individual. The stray cats sampled in this study were taken from areas where the community staying or working at the that area would feed them but still tested negative, meaning that minimal contact between individuals with stray cats were insufficient for the humans to transmit the virus to the cats. According to Kannekens-Jager et al., cats and dogs from households with multiple COVID-19 positive individuals showed higher prevalence of SARS-CoV-2 infection as compared to animals from households that only had one COVID-19 positive individual. This shows transmission from humans to cats is possible but requires high exposure to a COVID-19 positive individual, thus, it unlikely for a stray cat with minimal exposure to become infected with SARS-CoV-2.

Even though the result of this study is supported by previous studies, there were limitations for this study including a small sample size due to the limited time given for the research. Based on the sample size calculation formula, $n = \frac{z^2 \times \hat{p}(1-\hat{p})}{\varepsilon^2}$ at least 385 samples must be taken to achieve a confidence level of 95% of the prevalence of SARS-CoV-2 infections in stray cats. However, this can still be used as a preliminary study for further research to be done on a larger sample size.

In addition, non-specific bands were found in the results that were not able to be addressed due to the time constraint of this research. According to Bio-Rad Laboratories, non-specific bands may be produced due to cycling times and temperatures such as too many cycles used, extension time set for too long, annealing

time set for too long, annealing temperature set too low or thermal cycler ramping speed set too slow. PCR components may have also caused this with impurities within the primer, excessive primer added, incorrectly designed primer, impure dNTPs used, too much Mg^{2+} added or impure water used. The exact cause was not identified but is believed to be due to the annealing temperature being too low.

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In conclusion, based on this study, SARS-CoV-2 infection was not identified and is not present in stray cats from Kota Bharu, Kelantan. Only one of the cats sampled showed mild respiratory signs but was tested negative. Transmission of SARS-CoV-2 from humans to cats is only seen in cases of high exposure time, hence, the general public should not be worried about any stray cats being a source of infection of the disease.

6.2 Recommendation and Future Work

As for the recommendation and future work, an increase in sample size to the ideal sample size based on the statistical calculation should be done in order to achieve a result with 95% confidence level. A collaboration with large hospitals and veterinary clinics in Malaysia should be done to obtain the prevalence of the disease in animals throughout Malaysia. Additionally, other species such as stray dogs and birds can also be included as subjects of interest as there is also a large population of strays of these species in Malaysia. Furthermore, ELISA can be done hand in hand with RT-PCR to confirm the exposure of these strays to SARS-CoV-2 virus.

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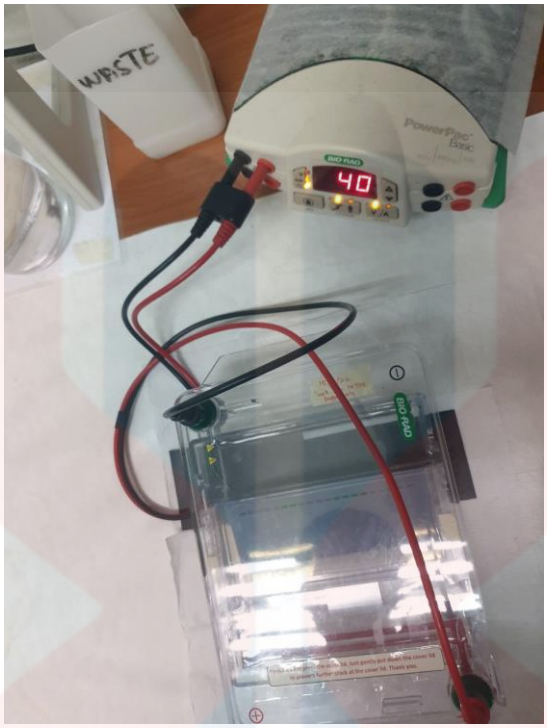
APPENDICES

Cat Number	Age Group	Sex	Physical Examination Findings
1	1-5 Years Old	M	Pregnant
2	<1 Year Old	F	Presence of flea dirt on hair coat; Presence of ear wax in both ears
3	1-5 Years Old	M	Skin tenting 3 seconds; Presence of circular, wet, not well-demarcated wound at the head area; Bilateral submandibular, right prescapular, bilateral popliteal lymph node enlargement;
4	1-5 Years Old	F	No abnormal findings
5	1-5 Years Old	M	Rough hair coat; Presence of lacerated wound caudal to the left ear base; Left submandibular lymph node enlargement; Presence of tear stain bilaterally; Presence of ear wax in left ear
6	1-5 Years Old	F	No abnormal findings; Polydactyl
7	1-5 Years Old	M	Presence of lice on haircoat; Mild left popliteal lymph node enlargement; Presence of soft mucoid faeces on perineal area
8	1-5 Years Old	M	Presence of lice on haircoat; Mild bilateral submandibular lymph node enlargement; Tachycardic

9	1-5 Years Old	F	Presence of fleas on skin; Tear stain present on both eyes; Mild crusty skin lesion on both ear pinna; Dirty ear canals filled with earwax
10	1-5 Years Old	M	Crusty skin lesion and alopecia on both ear pinna tips; Gingivitis on upper arcades; Presence of indolent ulcer
11	1-5 Years Old	F	Presence of lice on haircoat; Bilateral submandibular and popliteal lymph node enlargement
12	< 1 Year Old	M	Pale pink and tacky mucous membrane; Presence of both fleas on skin; Both ears are dirty and filled with earwax
13	1-5 Years Old	F	Bilateral submandibular lymph node enlargement
14	< 1 Year Old	F	Presence of flea and lice on haircoat
15	< 1 Year Old	M	Presence of flea dirt on haircoat; Gingivitis on both upper and lower arcades; Crusty skin lesion on both ear pinna

Appendix A: The age, sex and physical examination findings of the cats sampled.

Appendix B: The strays cat kept in a cage with food, water, litter and a mat provided.



Appendix C: Bio-Rad Gel Electrophoresis Chamber used in this research