MOLECULAR DETECTION OF *Mycoplasma haemofelis* IN CATS IN SELECTED DISTRICTS IN KELANTAN

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A RESEARCH PAPER SUBMITTED TO THE FACULTY OF VETERINARY MEDICINE, UNIVERSITI MALAYSIA KELANTAN
IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR
THE DEGREE OF
DOCTOR OF VETERINARY MEDICINE

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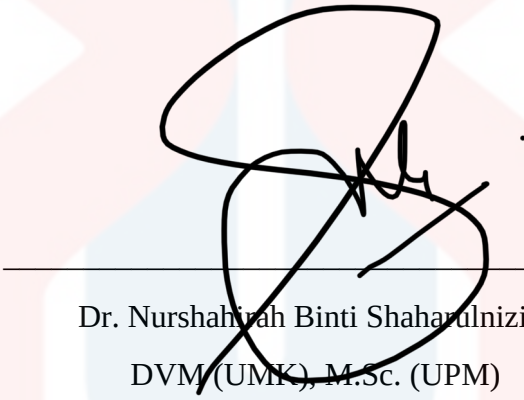
UNIVERSITI MALAYSIA KELANTAN

CERTIFICATION

This is to certify that we have read this research paper entitled **'Molecular Detection of *Mycoplasma haemofelis* in Cats in Selected Districts in Kelantan'** by Subashini A/P Ratha Pukallenthy, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course DVT 5436 – Research Project.



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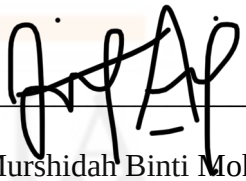

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Family

DVM 5 class of 2018/2022

Thank You

DEDICATIONS

I dedicate my dissertation work to my family members and many friends. A special appreciation to my loving parents, siblings and Manoj Chandara Rao.

I also dedicate this study to many of my lecturers who have braced me throughout the process. I will always acknowledge all of the things they have done, especially Dr. Nurshahirah Binti Shaharulnizim and Dr Murshidah Binti Mohd Asri for helping me develop my skills as a veterinary student.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT 5436 – Research Project.

This research is aimed to detect the presence of *Mycoplasma haemofelis* infection in cats in Kota Bharu and Bachok, Kelantan, Malaysia, using molecular method. Samples were collected from 18 household cats after obtaining consent from the Faculty of Veterinary Medicine at Universiti Malaysia Kelantan (UMK) and the Final Year Project (FYP) Committee (UMK/FPV/ACUE/FYP/003/2022). Whole blood samples were collected and then analysed using Polymerase Chain Reaction (PCR). In conclusion, the outcome of this research was all negative for all the 18 blood samples that was collected indicating that the household cats in Kota Bharu and Bachok, Kelantan are not infected with *Mycoplasma haemofelis*. This result was supported by absence of expected bands from the samples other than the positive control.

Keywords: *Mycoplasma haemofelis, cats, whole blood, Polymerase Chain Reaction*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Penyelidikan ini bertujuan untuk mengesan kehadiran jangkitan *Mycoplasma haemofelis* pada kucing di Kota Bharu dan Bachok, Kelantan, Malaysia, menggunakan kaedah molekul. Sampel diambil daripada 18 ekor kucing rumah selepas mendapat persetujuan daripada Fakulti Perubatan Veterinar Universiti Malaysia Kelantan (UMK) dan Jawatankuasa Projek Tahun Akhir (FYP) (UMK/FPV/ACUE/FYP/003/2022). Sampel darah telah dikumpul. dan kemudian dianalisis menggunakan Polymerase Chain Reaction (PCR). Kesimpulannya, hasil kajian ini adalah negatif bagi kesemua 18 sampel darah yang dikumpul menunjukkan kucing rumah di Kota Bharu dan Bachok, Kelantan tidak dijangkiti *Mycoplasma haemofelis*. Keputusan ini disokong oleh ketiadaan jalur jangkaan daripada sampel selain daripada kawalan positif.

Kata kunci: *Mycoplasma haemofelis, kucing, darah, Polymerase Chain Reaction*

1.0 Introduction

Hemotropic mycoplasmas are bacterial entities with no cell walls which adhere on the surface of red blood cells and develop there. They are gram-negative and non-acid-fast, and cultivating them on artificial media has failed despite several attempts (Greene, 2012). For many years, these epicellular organisms were classified as rickettsia, belonging to the genera Eperythrozoon and Haemobartonella. Sequencing of the 16S rRNA (ribosomal RNA) and Ribonuclease (RNase) P RNA genes, on the other hand, indicates that they are mycoplasmas (Greene, 2012). These hemotropic mycoplasmas have been given the generic name Hemoplasmas. DNA and RNA are found in hemotropic mycoplasmas, which replicate through binary fission. They can be rod-shaped, spherical, or ring-shaped and can be found individually or in chains on red blood cells.

The cat can be infected by three hemoplasmas, which are *Mycoplasma haemofelis*, " *Candidatus Mycoplasma haemominutum* ", and " *Candidatus Mycoplasma turicensis* ". The sequences of 16S rRNA and RNase P RNA genes in these organisms are about 83% identical (Greene,2012). Mycoplasmas that infect a wide spectrum of mammals are known as hemotropic mycoplasmas. Although human infection is uncommon, there has been speculation that it is linked to an immunocompromised state (dos Santo *et al*., 2008). The study reported that a case of a *Mycoplasma haemofelis*– like infection in an HIV-positive patient co-infected with *Bartonella henselae*. Hemoplasmosis in human also has been reported in association with systemic lupus erythematosus in the USA and Crotia (Tasker *et al*., 2010).

Despite the fact that *Mycoplasma haemofelis* has been identified as a hemoparasite of cats over the world and has turned into a public health issue, research on the prevalence of diseases in cats in various parts of the world has been lacking (Aklilu *et al*., 2016). Since, there are less studies reporting on the prevalence of *Mycoplasma haemofelis* in Malaysia, as a result, this study was carried out to look at the occurrence and prevalence of *Mycoplasma haemofelis* in household cats in selected districts in Kelantan by using molecular detection which is Polymerase Chain Reaction (PCR).

2.0 Research problem

Tick-borne illnesses in cats are a global problem. The tropical and subtropical regions are known for their ideal climatic conditions, which help vectors survive and reproduce. However, in Kelantan there are only several studies that were done on this potentially zoonotic organism. As for that, the purpose of this study was to look at the occurrence and prevalence of *Mycoplasma haemofelis* in household cats by using molecular detection which is Polymerase Chain Reaction (PCR) in selected districts in Kelantan.

3.0 Research questions

3.1 Is there any household cats with *Mycoplasma haemofelis* detected by using molecular method in Kota Bharu and Bachok, Kelantan, Malaysia?

4.0 Research hypothesis

4.1 Household cats in Kota Bharu and Bachok, Kelantan, Malaysia can be detected with *Mycoplasma haemofelis* infection by using molecular method.

5.0 Objectives

5.1 To detect the presence of *Mycoplasma haemofelis* infection in household cats in Kota Bharu and Bachok, Kelantan, Malaysia, using molecular method.

6.0 Literature review

6.1 Characteristics of *Mycoplasma haemofelis*

Mycoplasmal pathogen (hemoplasma) which is *Mycoplasma haemofelis* causes hemolysis in cats. Nonetheless, *M. haemofelis*, like other mycoplasmas, it is linked to Gram-positive bacteria and lacks a cell wall., from which it developed through a reduction in the size of the genome. The hemoplasmas are distinguished from other mycoplasmas by their distinct erythrocyte tropism also in addition when compared to the nearest equivalent mucosal mycoplasma species, their 16S rRNA genes had a low sequence similarity. (Santos *et al*.,2011).

6.2 Transmission of *Mycoplasma haemofelis*

The transmission of this disease can be by *M. haemofelis*-infected fleas that can spread parasites and cause sickness in a vulnerable cat which is considered as primary mode of transmission (Blue & French, 2016). Otherwise, infection with *M. haemofelis* has been spread through intraperitoneal and intravenous injections, as well as oral delivery of infected blood by experimentally. Next, in the absence of arthropod vectors, *M. haemofelis* is thought to be transmissible from queens with clinical illness to their new born offspring. Because experimental research studying this mechanism of transmission are

sparse, it is uncertain if this transfer occurs in utero, during parturition, or through nursing because there is lack of experiments on this (Greene,2012). Also, transfusion of blood from clinically healthy carrier cats can result in iatrogenic transmission of *M.haemofelis* (Greene,2012).

6.3 Clinical manifestation of *Mycoplasma haemofelis*

The severity of *M. haemofelis* disease varies, with some cats suffering from moderate anaemia with no clinical signs and others suffering from severe depression and anaemia that leads to death (Green,2012). Also, it can induce acute haemolytic anaemia, may also induce infected erythrocytes to commit suicide, either directly or by causing immune-mediated death of red blood cells (Santos *et al.*, 2011). A wide range of clinical symptoms, including anaemia, pyrexia, lethargy, and splenomegaly, characterise the illness, which, if left untreated, can result in mortality. *Mycoplasma haemofelis* is also known to be a pathogen when it is found in the presence of retroviruses such as the feline immunodeficiency virus (FIV), feline leukaemia virus (FeLV), or other devastating diseases (Santos *et al.*, 2011).

6.4 Detection of *Mycoplasma haemofelis* using Polymerase Chain Reaction

Polymerase chain reaction based molecular detection (PCR) of *Mycoplasma* spp. infection using the 16S rRNA gene of hemotropic *Mycoplasma* spp can be done. When compared to cytologic examination, it is more sensitive and specific (Watanabe *et al.*, 2008). Polymerase Chain Reaction analysis has proved as its own helpful and sensitive test. The expenses of examination, on

the other hand, is substantial, and the time required is not quick. According to multiple studies, direct PCR utilizing a template such as whole blood or excrement can detect a variety of gene abnormalities and infectious illnesses (Watanabe *et al.*, 2008).

7.0 Materials and methods

7.1 Ethics statement and permit approval

Students carried out the sampling procedures under the supervision of veterinarians, also with consent from the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (UMK), and the Final Year Project (FYP) Committee, with the ethics number UMK/FPV/ACUE/FYP/003/2022.

7.2 Study area and cat blood sampling

Eighteen blood samples were obtained from household cats around Kota Bharu and Bachok, Kelantan area. Relevant information such as gender, age, breed, and presence of ectoparasite, vaccination status and any clinical signs were recorded by using an online google form which was filled up by owners before the sample collection.

Blood sample about 2 to 3 ml was collected from the jugular vein or cephalic vein aseptically by using 23-gauge needle, disposable syringe and EDTA tube. During the blood collection, complete personal protective equipment (PPE) was worn, including a face mask and glove. All the collected samples were

transported in an icebox to the Faculty of Veterinary Medicine's molecular laboratory.

7.3 Sample collection

A towel wrap restraining approach was used where the animal was held and positioned for withdrawal of blood from jugular vein. Two to three ml of blood was collected from jugular vein aseptically by using 23-gauge needle, disposable syringe and EDTA tube. The blood tubes were then placed in a ice box and returned to the molecular laboratory and stored at the chiller at the temperature of 4°C.

7.4 Blood smear examination

A drop of blood was obtained and placed on a glass slide that had been cleaned. The drop of blood spread by using another slide. The other slide was pushed forward, allowing the blood to spread out and form a thin coating on the glass slide. After allowing the thin blood smear to dry, a cytological investigation was performed to observe the presence of the organism by using Diff Quick Stain.

7.5 DNA extraction

Mycoplasma haemofelis has a linear double stranded DNA genome. Hence, the organism must be extracted before being amplified by using Polymerase Chain Reaction techniques. A Genomic DNA Mini Kit for blood/cultured cells (Geneaid Biotech, Taiwan) which contains RBC lysis buffer, GT buffer, GB

buffer, W1 buffer, Wash buffer, Elution buffer, GD columns and 2ml collection tubes was used to purify the DNA from 18 whole blood samples that were collected at Molecular Laboratory, Faculty of Veterinary Medicine UMK.

As for the sample preparation, 300 µl of blood was transferred to a 1.5ml microcentrifuge tube. 900 µl of RBC lysis buffer was added then mixed by inversion. The tube was incubated at room temperature for 10 minutes. Then, it was centrifuged for 5 minutes at the speed of 3000 rft and the supernatant was discarded completely after the centrifugation. Again 100 µl of RBC lysis buffer was added to resuspend the leukocyte pellet and to proceed with cell lysis.

As for the cell lysis, 200 µl of GB buffer was added and the tube was shaken vigorously. The tube then incubated in the thermal block at 60℃ for 10 minutes to ensure the sample lysate is clear. During incubation, the tube was inverted for every 3 minutes. At the same time, elution buffer was preheated at the temperature of 60℃.

As for DNA binding step, 200 µl of absolute ethanol was added to lysate and then the tube was immediately mixed by shaking vigorously for 10 seconds. A GD column was in a 2 ml collection tube. The mixture was then transferred to the GD column then centrifuged at 14 000 rft for 5 minutes. After that, the 2 ml collection tube was discarded and then the GD column was placed in a new 2 ml collection tube. 400 µl of W1 buffer was added to the GD column and then centrifuged again at 14 000 rft for 1 minute. The flow through was then discarded and then the GD column was placed back in the 2 ml collection tube.

600 µl of wash buffer was added to the GD column. The tube was centrifuged at 14 000 rft for 1 minute and then again, the flow through was discarded. The GD column was placed back in the collection tube and centrifuged again for 3 minutes at 14 000 rft to dry the column matrix.

As the last step of the procedure, DNA elution. The dried GD column was transferred to a clean 1.5 ml microcentrifuge tube. 100 µl of pre heated elution buffer was added and centrifuged at 14 000 rft for 1 minute. The GD column was discarded and the 1.5 ml microcentrifuge tube with the purified DNA was kept in the - 80℃ freezer.

7.6 Detection of *Mycoplasma haemofelis* by Polymerase Chain Reaction (PCR)

Mh-F (5' GAGGGATAATTATGATAGTACTTCGTG 3') and Mh-R (5' CAATCTAGACATGTAGTATTCGGTG 3') primers were used to detect *Mycoplasma haemofelis* specifically. As for the polymerase chain reaction, pre-prepared master mix was used for all the samples and the PCR mixture was prepared according to table 1. Each run includes a positive control where the sample had sent for a DNA sequencing for *Mycoplasma haemofelis* and negative control which is nuclease free water.

PCR reactions were carried out using Eppendorf Master cycler® nexus under the following conditions: Initial denaturation at 72℃ for 45 seconds, 35 cycles of 45 seconds of denaturation at 94℃, 45 seconds of annealing at 55℃, 45 seconds of extension at 72 ℃ and a final extension step at 72℃ for 5 minutes.

Reagent	Volume per reaction (µL)	Volume for 22 reactions (µL)
Master mix	12.5	275
Forward primer	1	22
Reverse primer	1	22
Nuclease free water	5.5	121

Table 1 Polymerase Chain Reaction (PCR) components

7.7 Agarose Gel Electrophoresis

40 ml of TBE buffer was mixed with 0.8 g of agarose powder to prepare 2% of agarose gel in a Scott bottle. After that, the mixture was microwaved for 1–2 minutes, or until the agarose was completely dissolved. The agarose solution was then let to cool down to about 50℃ and 1 µL of Midori Green dye was added into the agarose solution. With the well comb in place, the agarose solution is poured into the gel tray. The gel was then left at room temperature for 30 minutes to solidify entirely. The agarose gel is then placed in the electrophoresis tank once it has set. The tank then filled with 1x TBE buffer until the gel is covered. The 1 Kb DNA ladder was loaded in the first lane of the gel. After that, the samples were loaded carefully in the additional wells of gel and last two wells with positive and negative control respectively. The gel was run at 100V for 40 minutes. Later, the gel was removed from the tank and DNA fragments was visualized using GelDoc™ EZ Imager (Bio-rad,USA) where the DNA fragment is usually referred as band due to their appearance on the gel. The expected product size for *Mycoplasma haemofelis* is 190bp.

8.0 Results

Between April to May 2022, 16 indexes participants from 14 households with 18 cats were enrolled in this research.

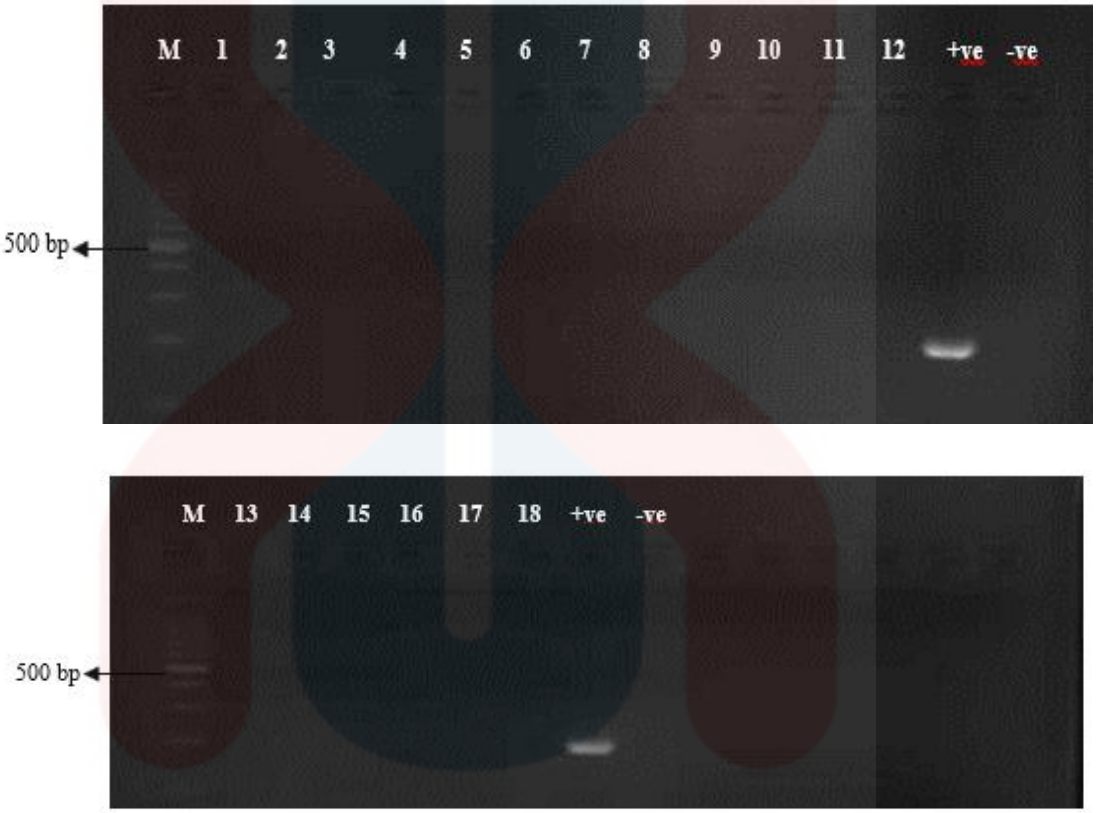

Figure 1: Detection of Mycoplasma haemofelis in cats by agarose gel electrophoresis where M represents DNA ladder, well 1 to 18 represents samples from the blood of cat, +ve positive and -ve negative control.

The 18 blood samples from cats that were collected were examined for Polymerase Chain Reaction. The findings are displayed in Figure 1. Figure 1 revealed negative outcomes from all the 18 samples.

9.0 Discussion

Ctenocephalides felis is the carrier of *Mycoplasma haemofelis*, a gram-negative bacterium. Giemsa stain or Diff Quick stain can be used in the examination of thin blood smear. The approach, however, is unreliable since the microscopic pathogen looks a lot like stain debris, protein precipitates, and Howell Jolly bodies (Watanabe *et al*.,2008). In this research, thin blood smear was done with the blood that was collected. There were no any significant findings from the procedure that was done. Watanabe (2008) stated that molecular detection of *Mycoplasma spp* by using Polymerase Chain Reaction is more useful, specific and sensitive. Thus, in this study Polymerase Chain Reaction was used to determine the presence of *Mycoplasma haemofelis* from the 18 household cats blood samples. All the samples gave a negative result. This indicate that the household cats are free of Mycoplasma infection.

There is a study which was conducted by Aklilu (2016) in stray cats at Kota Bharu, Kelantan. Seven out of 60 blood samples were positive for the presence of *Mycoplasma haemofelis*. This indicating that the prevalence of occurrence of this Mycoplasmal infection is high in the stray cats compared to household cats. This is because the household cats are kept in a good condition without any infestation of the vector such as cat fleas. Apart from that, mycoplasmal infection also can be transmitted through cat fight which is very common in outdoor male cats where they have such aggressive interactions (Kaewmongkol *et al*.,2020). Since, the cats in this study are owned, there is less likely for them to be infected.

The common clinical signs that can be observed in cats that have been infected by *Mycoplasma haemofelis* are lethargy, weight loss, anorexia, fever, thrombocytopenia, jaundice and also it is the cause of feline infectious anemia (Suksai *et al*.,2010). Physical examination was done before collecting from the household cats before

withdrawing blood. The cats that were presented in this case were all in good body condition, having a good appetite, appeared bright, alert and responsive. With these findings there are less likely for these cats to be infected. Also, the cats also had absented of fleas on their body. This can be an evident for them for not harbouring the disease.

Furthermore, in this study we could not get more blood samples due to cat's unwillingness to cooperate throughout the sampling process. Other than that, the negative finding of all 18 samples could be due to sampling technique. For example, the low pathogen load and degradation of the sample may cause false- negative findings as the blood was processed after three days been kept under 4℃. Next, there might be presence of blood clot or lyse of RBC while transferring the blood from the syringe to the EDTA tube. Also, there is a study stating that the when blood is collected in blood tubes containing an anticoagulant such as EDTA, *Mycoplasma haemofelis* may come off the surface of the erythrocyte (Aklilu *et al*.,2016). All these factors might be contributing in the negative results.

As for the limitation of this research is that the time constraint. With the limited time given, only less blood samples were able to collect for this study. Hence, the prevalence in household cats cannot be fully identified.

10.0 Conclusion

In conclusion, the polymerase chain reaction gave negative results for all the 18 samples, indicating that the household cats in Kota Bharu and Bachok, Kelantan are not infected with *Mycoplasma haemofelis*. This result was supported by absence of expected bands from the samples other than the positive control.

11.0 Recommendations and future work

Detection of *Mycoplasma haemofelis* in cats can be enhanced by increasing the sample size at least to 30 samples and incorporating particular sample testing criteria such as a cat exhibiting clinical symptoms. In addition, the scope of the research can be extended to learn more about endoparasites in cats. Furthermore, sampling methods and collection need to improve to increase the chance of getting positive outcomes. Also, sampling can be done in cats which are infested with fleas to obtain the positive results.

Appendix A

Covid-19 status in cats and risk factors related to the spreading of Covid-19 from humans to cats/ Status Covid-19 di dalam kucing dan faktor risiko yang menyebarkan Covid-19 dari manusia kepada kucing

This is a research project, and for this project to succeed, you will be needed to complete a short questionnaire. To complete this questionnaire, we will require you to spend 15 minutes answering the question in this survey.

Note that your information will not be released to anyone from our institution and remain anonymous. The data collected will only be available to researchers for analysis and interpretation. As this questionnaire requires your voluntary participation, you may withdraw from this research study any time you wish. Withdrawing from this study will not affect your legal rights. However, participation in this study will not involve any major risks whatsoever, physical or emotional. This study maybe will or will not directly benefit participants, but it will be of value for data collection. At the end of this survey, you will not receive any incentive or payment for your participation.

Ini adalah projek penyelidikan dan untuk projek ini berjaya, anda perlu melengkapkan soal selidik ini. Untuk melengkapkan soal selidik ini, kami memerlukan anda meluangkan masa 15 minit untuk menjawab soalan dalam tinjauan ini.

Harap maklum bahawa maklumat anda tidak akan disebarkan kepada sesiapa di luar institusi kami dan akan kekal tanpa nama. Data yang dikumpul hanya akan tersedia kepada penyelidik untuk analisis dan tafsiran keputusan. Oleh kerana soal selidik ini memerlukan penyertaan sukarela anda, anda boleh menarik diri daripada kajian penyelidikan ini pada bila-bila masa anda mahu. Menarik diri daripada tinjauan ini tidak akan menjejaskan hak undang-undang anda. Walau bagaimanapun, penyertaan dalam kajian ini tidak akan

Appendix A 1: The online Google form used in this study

UNIVERSITI MALAYSIA KELANTAN

Fakulti Perubatan Veterinar

RUJ. KAMI (*Our Ref*) : UMK/FPV/ACUE/FYP/003/2022
TARIKH (*Date*) : 26 FEBRUARY 2022

DR. NURSHAHIRAH BINTI SHAHARULKIZIM
Main Supervisor
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan

Dear Dr,

APPROVAL OF INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) TO CONDUCT RESEARCH INVOLVING ANIMALS

We are pleased to inform you that your application for approval to conduct research from Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Universiti Malaysia Kelantan has been approved. Please refer the table below for approval code:

APPROVAL CODE	*UMK/FPV/ACUE/FYP/003/2022*
TITLE	*MOLECULAR DETECTION OF Mycoplasma haemofelis IN CATS IN SELECTED DISTRICTS IN KELANTAN.*

2. Please be noted for the Final Year Project, you are responsible to supervise your student to conduct all animal-related procedures as stated during ethic application. The co-supervisor(s) for the project are encouraged to help with the procedures as well.

3. You are advised to always follow "3R" (REDUCE, REFINE, & REPLACE) and all animal ethics and animal welfare principles to reduce suffering in animal.

Thank you.

"RAJA BERDAULAT, RAKYAT MUAFAKAT, NEGERI BERKAT"
"WAWASAN KEMAKMURAN BERSAMA 2030"
"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

DR. NOR FADHILAH BINTI KAMARUZZAMAN
Chairman
Institutional Animal Care and Use Committee
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan

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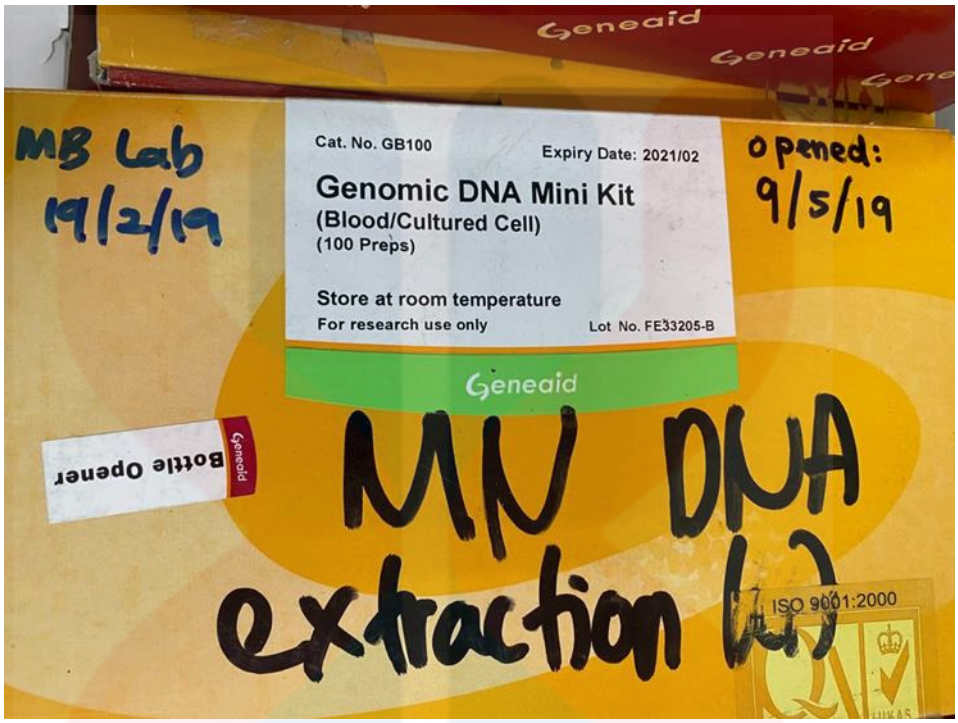
Appendix A 2: Approval form from Institutional Animal Care and Use Committee (IAUCC)

Appendix B



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References

Aklilu, E., Shaharulnizim, N., Francis, J. J., & Anurrdin, S. H. (2016). Molecular investigation of Mycoplasma haemofelis in stray cats in Kota Bharu, Kelantan. Tropical Biomedicine, 33(4), 608-612.

Case, J. B., Chomel, B., Nicholson, W., & Foley, J. E. (2006). Serological survey of vector-borne zoonotic pathogens in pet cats and cats from animal shelters and feral colonies. Journal of Feline Medicine and Surgery, 8(2), 111-117.

Cetinkaya, H., Haktanir, D., Arun, S., & Vurusaner, C. (2016). Molecular detection and prevalence of feline hemotropic mycoplasmas in Istanbul, Turkey. *Acta parasitologica*, *61*(1), 165-171.

dos Santos, A. P., dos Santos, R. P., Biondo, A. W., Dora, J. M., Goldani, L. Z., De Oliveira, S. T., ... & Messick, J. B. (2008). Hemoplasma infection in HIV-positive patient, Brazil. Emerging infectious diseases, 14(12), 1922.

Greene, C. E. (2006). Infectious diseases of the dog and cat (No. Ed. 3). WB Saunders\Elsevier Science.

Imre, M., Văduva, C., Dărăbuș, G., Morariu, S., Herman, V., Plutzer, J., ... & Imre, K. (2020). Molecular detection of hemotropic mycoplasmas (hemoplasmas) in domestic cats (Felis catus) in Romania. *BMC Veterinary Research*, *16*(1), 1-8.

Kaewmongkol, S., Lakhana, N., Sirinarumitr, T., Fenwick, S. G., & Kaewmongkol, G. (2020). Investigation of hemotropic Mycoplasma spp. genotypes in client-owned cats in Thailand. Veterinary Microbiology, 247, 108765.

Lashnits, E., Neupane, P., Bradley, J. M., Richardson, T., Thomas, R., Linder, K. E.,... & Breitschwerdt, E. B. (2020). Molecular prevalence of Bartonella, Babesia, and hemotropic Mycoplasma species in dogs with hemangiosarcoma from across the United States. PLoS One, 15(1), e0227234.

Persichetti, M. F., Solano-Gallego, L., Serrano, L., Altet, L., Reale, S., Masucci, M., & Pennisi, M. G. (2016). Detection of vector-borne pathogens in cats and their ectoparasites in southern Italy. *Parasites & Vectors*, *9*(1), 1-7. Santos, A. P., Guimaraes, A., do Nascimento, N. C., SanMiguel, P. J., Martin, S. W.,& Messick, J. B. (2011). Genome of Mycoplasma haemofelis, unraveling its strategies for survival and persistence. Veterinary research, 42(1), 1-16.

Ravagnan, S., Carli, E., Piseddu, E., Da Rold, G., Porcellato, E., Zanardello, C., ... & Capelli, G. (2017). Prevalence and molecular characterization of canine and feline hemotropic mycoplasmas (hemoplasmas) in northern Italy. *Parasites & vectors*, *10*(1), 1-7.

Suksai, P., Sangkachai, N., Chatsiriwech, J., Kanthasaewee, O., Sariya, L., & Chaichoun, K. (2010). Development of multiplex polymerase chain reaction for detection of feline hemotropic mycoplasma in blood and tissue specimens. Southeast Asian Journal of Tropical Medicine & Public Health, 41, 1447-1453.

Sykes, J. E. (2010). Feline hemotropic mycoplasmas. Journal of Veterinary Emergency and Critical Care, 20(1), 62-69.

Varanat, M., Maggi, R. G., Linder, K. E., & Breitschwerdt, E. B. (2011). Molecular Prevalence of B artonella, B abesia, and Hemotropic M ycoplasma sp. in

Dogs with Splenic Disease. Journal of veterinary internal medicine, 25(6), 1284-1291.

Watanabe, M., Hisasue, M., Souma, T., Ohshiro, S., Yamada, T., & Tsuchiya, R. (2008). Molecular detection of Mycoplasma haemofelis andCandidatus Mycoplasma haemominutum'Infection in cats by direct PCR using whole blood without DNA extraction. Journal of Veterinary Medical Science, 70(10), 1095-1099.

Willi, B., Boretti, F. S., Meli, M. L., Bernasconi, M. V., Casati, S., Hegglin, D., ... & Hofmann-Lehmann, R. (2007). Real-time PCR investigation of potential vectors, reservoirs, and shedding patterns of feline hemotropic mycoplasmas. *Applied and Environmental Microbiology*, *73*(12), 3798-3802.