MOLECULAR DETECTION OF LEPTOSPIRA SPECIES AMONG DOGS IN KELANTAN

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Molecular Detection of Leptospira Species among Dogs in Kelantan

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MOLECULAR DETECTION OF LEPTOSPIRA SPECIES AMONG DOGS IN KELANTAN

ABSTRACT

Leptospirosis is a bacterial disease that can infect many mammals including dogs. It carries the potential for zoonotic transmission, presenting a risk to humans and posing threats to public health. There has been a lack of studies conducted on canine leptospirosis in Kelantan, Malaysia. This study was conducted to detect the presence of *Leptospira* spp. among dogs in Kelantan by molecular detection. A total of 31 blood samples were collected from the dogs in Kelantan. After DNA extraction of blood samples, Polymerase Chain Reaction (PCR) targeting the 16S rRNA gene was performed followed by gel electrophoresis. Among the 31 sampled dogs, 54.84% were female, 45.16% were male, and most of them were adults age ranging from 1 to 6 years old by 67.74%. The findings revealed the absence of *Leptospira* spp. in the collected blood samples from dogs in Kelantan. Findings highlighting the absence of *Leptospira* have a significant impact on enhancing the understanding of local disease epidemiology, suggesting a potential minimal threat of canine leptospirosis. In conclusion, the prevalence of *Leptospira* spp., as determined by molecular detection through PCR among dogs in Kelantan, was zero.

Keywords: Leptospirosis, Dogs, PCR, 16S rRNA

PENGESANAN MOLEKUL SPESIES LEPTOSPIRA DI KALANGAN ANJING DI KELANTAN

ABSTRAK

Leptospirosis adalah penyakit bakteria yang boleh menjangkiti pelbagai mamalia termasuk anjing. Ia berpotensi untuk penularan zoonosis, membawa risiko kepada manusia dan mengancam kesihatan awam. Kajian yang dilakukan mengenai leptospirosis anjing di Kelantan, Malaysia adalah kurang. Kajian ini dilakukan untuk mengesan kehadiran *Leptospira* spp. dalam anjing di Kelantan melalui pengesanan molekul. Sebanyak 31 sampel darah telah diambil daripada anjing di Kelantan. Selepas pengekstrakan DNA daripada sampel darah, *Polymerase Chain Reaction* (PCR) yang menyasarkan gen 16S rRNA telah dijalankan dan diteruskan dengan *gel electrophoresis*. Di antara 31 sampel anjing, 54.84% adalah betina, 45.16% adalah jantan, dan kebanyakannya (67.74%) adalah anjing dewasa berumur antara 1 hingga 6 tahun. Penemuan mendedahkan *Leptospira* spp. adalah tiada dalam sampel darah yang dikumpul daripada anjing di Kelantan. Kesimpulannya, prevalens *Leptospira* spp. yang ditentukan dengan pengesanan molekul melalui PCR di kalangan anjing di Kelantan adalah kosong.

Kata kunci: Leptospirosis, Anjing, PCR, 16S rRNA

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TABLE OF CONTENTS

		PAGE
ORIGINAL LITERARY WORK DECLARATION		ii
ABSTRACT		iii
ABSTRAK		iv
ACKNOWLEDGEMENTS		v
TABLE OF CONTENTS		vi
LIST OF TABLES		vii
LIST OF FIGURES		viii
LIST OF ABBREVIATIONS		iv
LIST OF SYMBOLS		x
CHAPTER 1 INTRODUCTION		
1.1	Research Background	1
1.2	Problem Statement	2
1.3	Research Question	2
1.4	Research Hypothesis	2
1.5	Research Objectives	2
CHAPTER 2 LITERATURE REVIEW		
2.1	Overview of Leptospirosis	3
2.2	Prevalence of Leptospirosis in Malaysia	4
2.3	Prevalence of Canine Leptospirosis	4
2.4	Laboratory Diagnosis of Canine Leptospirosis	5
CHAPTER 3 METHODOLOGY		
3.1	Ethical Approval	7
3.2	Study Area and Target Population	7
3.3	Acquiring Consent and Subject Medical History	7
3.4	Sample Collection	8
3.5	DNA Extraction	8
3.6	Polymerase Chain Reaction (PCR)	10
3.7	Gel Electrophoresis	11
CHAPTER 4 FINDINGS AND DISCUSSION		
4.1	Demographic Data of Dogs	12
4.2	Result of PCR	13
4.3	Discussion	14
CHAPTER 5 CONCLUSION AND RECOMMENDATION		17
REFERENCES		18
APPENDICES		20

LIST OF TABLES

NO.		PAGE
3.1	Samples collected according to districts in Kelantan	7
3.2	Calculation of components required for PCR	10
4.1	Demographic data of dogs sampled in Kelantan	12

LIST OF FIGURES

NO.		PAGE
4.1	Gel electrophoresis visualisation of the 16S rRNA gene amplification for the dog blood samples. 'M': 100 bp DNA ladder, 1 – 31: PCR products of the samples, '-ve': negative control, and '+ve': positive control	13

LIST OF ABBREVIATIONS

bp	Base pair
CDC	Centers for Disease Control and Prevention
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HPV	Veterinary Teaching Hospital
IACUC	Institutional Animal Care and Use Committee
MAT	Microscopic agglutination test
MOH	Ministry of Health
NFW	Nuclease-free water
NIL	Not in list
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
rRNA	Ribosomal RNA
TAE	Tris-acetate-EDTA
UMK	Universiti Malaysia Kelantan
UTD	Up to date
FPV	Faculty of Veterinary Medicine

LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
g	Gram
>	Greater than
≥	Greater than or equal to
<	Less than
ml	Millilitre
µl	Microlitre
µM	Micromolar
n	Number of individuals in the samples size
× *g*	Times gravity
™	Trademark sign
®	Registered trademark sign
V	Volt

CHAPTER 1

INTRODUCTION

1.1 Research Background

Leptospirosis is a bacterial disease that infects many mammals including dogs, cattle, horse, sheep, goat and pigs. It is a zoonotic disease that can be transmitted from animals to humans. Leptospira is an aerobic, Gram-negative bacteria characterized by its corkscrew-shape with hooked ends. *Leptospira* spp. can be classified into three monophyletic groups pathogenic, intermediate and saprophytic and there are more than 65 known species within the Genus *Leptospira* (Arent *et al.,* 2022).

The bacteria can be transmitted to humans by either direct or indirect transmission. Leptospirosis can result in various clinical signs in dogs from mild, subclinical infection to multiple organ failure and death. The infected animals can shed *Leptospira* in their urine after colonization in proximal tubules of the kidney (Yamaguchi *et al.,* 2018).

The diagnostic workup for the detection of leptospirosis includes culture isolation and identification, molecular and serological diagnostic tests. The molecular test involves polymerase chain reaction (PCR) and the serological test involves microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA).

In Malaysia, majority of studies on canine leptospirosis were conducted in Selangor and Johor states and involved urban areas. Dogs enrolled in these studies were either pets, sheltered, working or stray. The majority of studies collected samples from healthy dogs and few studies collected from dogs with liver and kidney disease. By culture isolation and PCR, *Leptospira interrogans* were the commonly detected species for samples from dogs (Kurilung *et al.,* 2017).

1.2 Problem Statement

Over time, the primary focus concerning the increased risk of human leptospirosis has shifted towards frequent and prolonged interaction with animals, particularly dogs. Leptospirosis burden is most prevalent in tropical regions worldwide, especially in developing countries like Malaysia. Research on leptospiral infection in dogs in Malaysia is scarce with some studies limited in Selangor and Johor state. There are no studies on canine leptospiral detection that have been conducted in Kelantan. Hence, this study aims to determine the prevalence of leptospirosis and identify the *Leptospira* species circulating among dogs in Kelantan.

1.3 Research Questions

i. What is the prevalence of leptospirosis among dogs in Kelantan?

ii. What are the species of *Leptospira* detected among dogs in Kelantan?

1.4 Research Hypothesis

i. There is a high prevalence of leptospirosis among dogs in Kelantan, Malaysia.

ii. The most prevalent *Leptospira* spp. that can be detected from dogs in Kelantan is *Leptospira interrogans*.

1.5 Research Objectives

i. To determine the prevalence of leptospirosis among dogs in Kelantan.

ii. To identify the most prevalent *Leptospira* spp. in dogs in Kelantan.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Leptospirosis

Leptospirosis is a zoonotic infectious disease of public health importance that caused by pathogenic *Leptospira* species. Developing and tropical countries, due to their tropical climate, agricultural practices, rapid urbanization and close contact between humans and animal reservoirs provide a favorable environment for the survival and transmission of *Leptospira*. The Genus *Leptospira* belongs to the family *Leptospiraceae* and is divided into three monophyletic groups namely saprophytes, intermediates and pathogens (Evangelista & Coburn, 2010). The most widely spread group is saprophytes, which are free-living non-pathogenic organisms that are present in natural environmental sources whereas the intermediate clade of *Leptospira* are present in both human and animal hosts resulting in mild clinical manifestations. Pathogenic *Leptospira* species is capable of infecting every mammal, causing general illness in susceptible hosts either in animals or humans. There are more than 65 recognized *Leptospira* spp. with over 250 identified serovars up to date (Arent *et al.,* 2022).

Almost all mammalian species, either wild or domestic can harbour *Leptospira* with or without any clinical symptoms. Animals stand in as maintenance hosts and accidental or incidental hosts with regard to leptospirosis transmission (Levett, 2001). In endemic countries, rodents are the main source of leptospirosis and are thus known to be the primary vector. Livestock animals like cattle, buffaloes, goats and pigs, pet animals such as dogs and wild animals are identified as potential reservoir animals of *Leptospira*. Humans acquire the organisms either through direct contact with the reservoir animal or indirectly via interaction with contaminated water sources or moist soil contaminated by urine of the carrier animal (Evangelista & Coburn, 2010).

2.2 Prevalence of Leptospirosis in Malaysia

Leptospirosis is an endemic disease in Malaysia that occurs in both urban and rural areas, where the unique tropical ecosystems contribute to the complex dynamics of its transmission. Benacer *et al.* (2016) states the heavy rainfall and frequent flooding, especially during the monsoon season in Malaysia, increase the incidence of leptospirosis. Ministry of Health Malaysia (2015) states the incidence rate of human leptospirosis cases in Malaysia increases from 1.03% in year 2004 to 30.2% in year 2015. The predominant *Leptospira* species causing human leptospirosis in Malaysia are *Leptospira interrogans* and *Leptospira kirschneri* (Philip *et al.,* 2020).

There are 37 serovars of *Leptospira* from 13 different serogroups have been identified, with a substantial portion originating from rodents in Malaysia (Benacer *et al.* 2013). Although there are extensive studies on leptospirosis in Malaysia, the actual disease burden remains underestimated.

2.3 Prevalence of Canine Leptospirosis

Several studies have investigated the prevalence and characterization of leptospirosis in dogs. According to the Centers for Disease Control and Prevention (2019), the incubation period of leptospirosis in dogs ranges from 5 to 14 days, but can be less than that or more than 30 days.

Though there is a commercial *Leptospira* vaccine for dogs in Malaysia, Rahman *et al.,* (2021) reported that both vaccinated and unvaccinated dogs can shed *Leptospira* in the urine of dogs. Nevertheless, the actual burden of dogs as a potential zoonotic reservoir for leptospirosis transmission is poorly reported and the overall influence to cause human leptospirosis is still poorly documented.

In a recent investigation by Rahman *et al.* (2021), the presence of *L. interrogans, L. borgpetersenii, L. kirschneri, and L. kmetyi* was revealed as pathogenic species among dog samples. Additionally, a study conducted by Khor *et al.* (2016) focused on *Leptospira* serovars in canines in Malaysia identified positive samples for *L. interrogans* serovar Bataviae, a serovar not covered by the local canine vaccination program.

Goh *et al.* (2021) determined into the seroprevalence of leptospirosis in stray dogs in the Klang Valley, Selangor state of Malaysia. Their findings indicated a high seroprevalence of *Leptospira* antibodies, with 32% of the samples testing positive. This suggests a significant exposure of stray dogs to the bacteria within the local environment, with all positive samples pointing to the presence of *L. interrogans*.

Dogs infected with *Leptospira* spp. exhibit a range of clinical signs such as inappetence, fever, anorexia, vomiting, icterus and usually with renal or hepatic dysfunction or both, together with hemorrhagic and pulmonary disorders. The study by Rahman *et al.* (2021) also involves sampling from patient dogs with kidney or liver disease or both. The samples were found to be positive for *L. interrogans* serovars Bataviae, Icterohaemorrhagiae and Australis. Moreover, the same study detected *L. borgpetersenii* serovar Javanica and *L. kirschneri* serovar Grippotyphosa.

2.4 Laboratory Diagnosis of Canine Leptospirosis

The laboratory diagnostic workup for the detection of leptospirosis includes culture isolation and identification, molecular and serological tests. The molecular test includes polymerase chain reaction (PCR) and the serological test involves microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). The gold standard for leptospirosis diagnosis is MAT, as it can further identify the specific serovar of the *Leptospira* species.

The PCR assay is a molecular technique used to detect the presence of *Leptospira* DNA in samples. It is a highly sensitive and specific test that amplifies and identifies the genetic material of the bacteria. In a study by Martin *et al.* (2022), the overall sensitivity of PCR using samples from dogs was 52.4% and its specificity was 100%.

Primers for PCR assays have been developed targeting either housekeeping genes that are present in all pathogenic, saprophytic and intermediate *Leptospira* species or specific genes that are only encoded in pathogenic species (Waggoner and Pinsky, 2016). Among housekeeping genes, 16S Ribosomal RNA (rRNA) rrs, secY, gyrB are widely used in PCR assays while lipL32, ligA, ligB, flaB and lfb1 are examples of pathogen specific genes.

PCR can be performed using various types of samples including blood, urine or tissue. It is useful in the diagnosis during the early stages of infection known as the leptospiraemia phase, when the bacteria may still be present in the bloodstream (Miotto *et al.,* 2018). PCR provides rapid results and can be helpful for early diagnosis and timely initiation of treatment. However, its limitations include the need for specialized laboratory equipment and expertise. The test may also not be widely available in all laboratory settings.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Ethical Approval

This study obtained ethical approval from the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (Appendix A) under approval code (UMK/FPV/ACUE/RES/001/2023).

3.2 Study Area and Target Population

The target population for this study was dogs in Kelantan, Malaysia. There were 31 dogs randomly included from the four districts in Kelantan which are Kota Bharu, Bachok, Pasir Mas and Kuala Krai (Table 3.1).

Table 3.1: Samples collected according to districts in Kelantan

No.	Districts	No. of samples collected
1.	Kota Bharu	14
2.	Bachok	11
3.	Kuala Krai	4
4.	Pasir Mas	2
	TOTAL	**31**

3.3 Acquiring Consent and Subject Medical History

Prior to blood sample collection, a brief explanation of the study and the procedure was explained to the owners. Once the owner agreed, consent form and contact information was signed and collected (Appendix B). Information regarding the patient's signalment, management and medical history was obtained using the study tool (Appendix C). Collected data were tabulated for further analysis (Appendix D).

3.4 Sample Collection

A total of 31 blood samples were collected from dogs in Kelantan, Malaysia from July to November 2023. The blood was collected from cephalic vein (venepuncture) aseptically following universal precautions into a EDTA tube. All collected samples were properly labelled and temporarily stored in a cooler bag. They were transported to the laboratory in FPV UMK and stored at -20°C before further processing.

3.5 DNA Extraction

DNA extraction of the blood samples was conducted using a commercial DNA extraction kit (Geneaid gSYNC^{TM}) following the manufacturer's instructions. All the required consumables (1.5 ml microcentrifuge tubes, micropipette tubes and PCR tubes) were sterilized by autoclaving prior to the DNA extraction procedure. EDTA tubes were taken out of storage and allowed to thaw at room temperature for 30 minutes before extraction.

First, 200 µl of each blood sample was transferred to a 1.5 ml microcentrifuge tube. For the insufficient blood, the volume was adjusted to 200 µl with phosphate-buffered solution (PBS). Next, 20 µl of proteinase K was added and mixed by pipetting. The prepared samples were incubated at 60°C for 10 minutes.

For cell lysis, 200 µl of GSB buffer was added to the incubated samples and then shaken vigorously with a vortex mixer. The mixed samples were incubated at 60°C for 10 minutes. During incubation, the required volume of elution buffer (100 µl per sample) was transferred into a 1.5 ml microcentrifuge tube and heated to 60°C for the last phase of the DNA extraction. As preparation for the next step, a GS column was placed in a 2 ml collection tube for each sample.

For DNA binding, the sample lysate was added with 200 µl of absolute ethanol and the lysate was mixed immediately by shaking vigorously for 10 seconds. The mixture was transferred to the prepared GS column and was centrifuged at 14,000 x g for 1 minute. The 2 ml collection tube containing the flow-through was discarded, and the GS column was transferred to a new 2 ml collection tube.

For the washing step, the GS column was added with 400 µl W1 buffer and centrifuged at 14,000 x g for 30 seconds. The flow-through was discarded and the GS column was re-inserted into the 2 ml collecting tube. The GS column was loaded with 600 µl of wash buffer and centrifuged for 30 seconds at 14,000 x g. The flow-through was then discarded, and the GS column was re-inserted into the 2 ml collecting tube. The GS column was then centrifuged again for 3 minutes at 14000 x g to dry.

The dried GS column was transferred to a clean 1.5 ml microcentrifuge tube for DNA elution. Next, 100 µl of pre-heated elution buffer was added in the centre of the column. The GS column was let to stand for 5 minutes to allow the elution buffer to be completely absorbed. The GS column was centrifuged again for 30 seconds at 14000 x g to elute the purified DNA. The extracted DNA samples were stored at -20°C.

3.6 Polymerase Chain Reaction (PCR)

The molecular detection of *Leptospira* spp. was conducted by conventional PCR targeting the 16S rRNA gene of *Leptospira* spp. and expected product size was 330 (base pair) bp. The 16S rRNA gene sequence of the forward primer was 5'-GGC GGC GCG TCT TAA ACA TG-3' and the reverse primer was 5'TCC CCC CAT TGA GCA AGA TT-3' (Merien *et al.* 1992).

A 25 µl of PCR reaction mixture containing 12.5 µl of PCR master mix (GoTaq®), 1 µl of 10µM forward primer, 1 µl of 10µM reverse primer and 5.5 µl nuclease free water was added into a 0.2 ml PCR tube. For convenience, they were calculated by multiplying to 34 units, which included 31 for sample DNA, 1 for positive control, 1 for negative control and 1 extra unit (Table 3.2). The calculated PCR components were prepared into a 1.5 microcentrifuge tube. Next, 20 µl of the prepared PCR component was transferred to 33 units of PCR tubes. Then, 5 µl of each sample DNA template was added to 31 units of PCR tubes. For the last two PCR tubes, 5 µl of positive control of *Leptospira* spp. and 5 µl of nuclease-free water (NFW) as negative control were added, respectively.

Table 3.2: Calculation of components required for PCR

PCR Components	1 unit (µl)	34 units (µl)
Master Mix	12.5	425
Forward Primer	1	34
Reverse Primer	1	34
Nucleus-Free Water (NFW)	5.5	187
Sample DNA	5	-
TOTAL	**25**	-

PCR amplification was conducted by thermal cycler. The initial denaturation was set at one cycle of 95°C for 5 minutes. Next, 35 cycles were set for denaturation at 95°C for 30 seconds, annealing at 60.4°C for 30 seconds, and the extension at 72°C for 60 seconds. The final cycle of extension was set at 72°C for 5 minutes.

3.7 Gel Electrophoresis

The amplified products were evaluated by agarose gel electrophoresis. First, 1.2 g of 1.5% agarose powder was weighed and mixed with 80 ml of 1x TAE buffer in a schott bottle. Next, the mixture was microwaved for a minute until the 1.5% agarose powder was completely dissolved. A 1 µl of Midori green dye was added into the heated agarose solution. A 30-well comb and a 15-well comb were arranged into a gel tray, and the solution was poured carefully into the gel tray. The gel was left for 40 minutes at room temperature to solidify.

After solidification, the wells combs were removed. The agarose gel was placed in an electrophoresis tank and filled with 1x TAE buffer until the gel was fully covered. A 5 µl of 100 bp DNA ladder was loaded into the first well of the gel. Next, the other wells were loaded with the PCR products. The last two wells were loaded with the positive control and negative control.

The gel electrophoresis was conducted at 100V for 45 minutes. After that, the gel was removed from the tank and transferred to GelDoc™ EZ Imager. The gel imaging system visualizes the DNA fragment as band following the gel present at the level of 330 bp.

CHAPTER 4

FINDINGS AND DISCUSSION

4.1 Demographic Data of Dogs

Table 1 shows the demographic data of enrolled animals in the study. Of 31 dogs sampled, 54.84% were female and 45.16% were male. Most of them were adults aged ranging from 1 to 6 years old (67.74%), 21.9% were young which was less than 1 year old, and 19.35% were a senior dogs aged more than 6 years old. Furthermore, 48.39% of the dogs were managed indoors and the remaining 51.61% dogs were managed outdoors. Among 31 dogs, 15 were vaccinated and 16 dogs were unvaccinated.

Table 4.1: Demographic data of dogs sampled in Kelantan (n = 31)

	No. of dog samples	Percentage (%)
Age		
Young (<1 year old)	4	12.90
Adult (1 to < 6 years old)	21	67.74
Senior (≥ 6 years old)	6	19.35
Sex		
Male	14	45.16
Female	17	54.84
Breed		
Mongrel	17	54.84
Small	7	22.58
Medium	7	22.58
Management		
Indoor	15	48.39
Outdoor	16	51.61
Vaccination Status		
Up to date (UTD)	15	48.39
Not in list (NIL)	16	51.61
Health Status		
Healthy	26	83.87
Kidney disease	1	3.23
Other diseases	4	12.90

4.2 Result of PCR

The amplification of 16S rRNA gene of *Leptospira* spp. showed that all of the samples were negative for PCR. Figure 4.1 indicates the absence of any band in the sample lanes on the same level as the positive control band. Thus, all 31 samples were negative for the leptospiral 16S rRNA gene. The only band present is at the positive control lane, which indicates the presence of *Leptospira* spp. DNA at level of 330 bp.

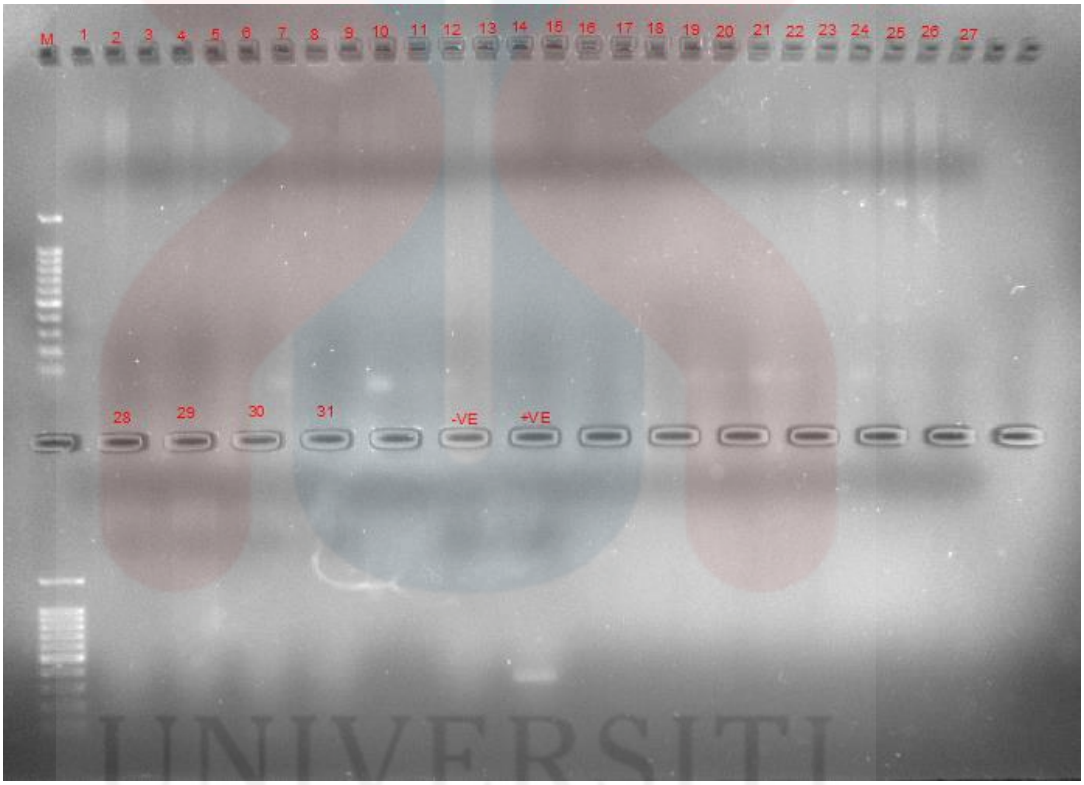

Figure 4.1: Gel electrophoresis visualisation of the 16S rRNA gene amplification for the dog blood samples. 'M': 100 bp DNA ladder, '1 – 31': PCR products of the samples, '-ve': negative control, and '+ve': positive control

4.3 Discussion

Canine leptospirosis includes multiple species of *Leptospira* and subsequent serovars. This is the first study conducted to investigate the prevalence of *Leptospira* spp. among dogs in Kelantan. Based on the result of the PCR, all the blood samples collected from the dogs in Kelantan were negative for *Leptospira* spp., thus the prevalence of canine leptospirosis by molecular detection was 0%. However, the results need to be interpreted carefully due to factors such as small sample size, lack of sample type, and inadequate diagnostic methods.

The sample size of the current study (n = 31) was relatively small and not sufficient for a representative result. A study by Rahman *et al.* (2021) had a larger sample size of 124 pet dogs, in which 33.9% were detected positive for the pathogenic *Leptospira* spp. in the whole blood samples. The small sample size of dogs in Kelantan is due to low dog population and dog shelters available in Kelantan compared to other states in Malaysia. Even the initial location of sampling at Bachok and Kota Bharu districts was insufficient, as more samplings were needed at other locations like Kuala Krai district (Table 3.1).

The diagnosis with PCR alone is insufficient, as it is not the gold standard for leptospirosis detection. The gold standard of leptospiral detection is the microscopic agglutination test (MAT). There are other diagnostic approaches for diagnosing leptospirosis which are serological detection by enzyme-linked immunosorbent assay (ELISA), and culture isolation. However, this study was only focused on the molecular detection by PCR.

In this study, the only collected sample type was blood. The urine samples have an additional advantage of being used as a sample during the leptospiruria phase, when the bacteria are shed from the urine of the infected dogs as well as during the stage of chronic carrier. A study by Llewellyn *et al.* (2016) found that 3 out of 200 urine samples were found to be positive for PCR, which results in a urinary shedding prevalence of 1.5% in healthy dogs.

Thus, it is suggested to increase in sample size, a combination of multiple detection methods of PCR, MAT and ELISA, and the addition of urine samples to overcome the likelihood of false negative results and for a proper interpretation before conclusion.

There were planned steps in case if there were any positive results for 16S rRNA PCR. The positive result of PCR was planned to be continued by using LipL32 primer for the detection of pathogenic species of *Leptospira*. The subsequent plan was to send the samples with positive PCR results for Sanger sequencing. Due to the negative results of the sample in this study, further sample processing was discontinued after the visualisation of the 16S rRNA gene amplification of the PCR products.

In terms of demographic data of dogs sampled in Kelantan (n = 31), there were several parameters of the dogs that were acquired (Table 4.1). Only dogs with owners and from shelters were sampled, as stray dogs were impractical to be caught and riskier for blood collection.

The sampled dogs with up-to-date vaccination were 48.39%, while the others with unavailable vaccination status were 51.61%. A study by Midence *et al.* (2012) states that all dogs sampled at all time points after vaccination were negative on PCR. Thus, the dogs vaccinated with *Leptospira* spp. were already immunized which contributes to the negative results in the PCR.

Most of the dogs sampled were healthy (26 dogs) and out of four other dogs with diseases, only one of them had kidney-related disease. However, this dog did not show clinical signs suggestive of leptospirosis such as icterus, fever and vomiting. A study by Rahman *et al.* (2021) found that 33.9% of the whole blood and 31.9% urine samples from dogs with pet dogs diagnosed with kidney and liver diseases were positive for pathogenic *Leptospira* spp. by PCR. Thus, enrolment of healthy dogs and dogs without any suggestive clinical signs of leptospirosis could be one of the major reasons for the negative results in the current study.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

In conclusion, the prevalence of *Leptospira* species by molecular detection of PCR among dogs in Kelantan was zero. However, it is crucial to recognize that a negative outcome does not rule out the potential for leptospirosis infections among dogs in Kelantan. The limitations of the current study were the small sample size, lack of sample type, and inadequate diagnostic methods which will be contributed to the interpretation of results carefully. Furthermore, most dogs sampled were healthy without any suggestive clinical signs of leptospirosis and some dogs were vaccinated with *Leptospira* spp. that could lead to the negative results in the current study.

As recommendation, additional investigations are suggested by targeting a larger sample size that encompasses urine as a specimen other than blood including other diagnostic methods such as MAT, ELISA and culture isolation to determine the prevalence of leptospirosis among dogs in Kelantan. Additionally, enrolment of dogs with suggestive clinical signs of leptospirosis, and unvaccinated dogs can be considered for further detection of *Leptospira* spp. in the future study.

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A copy of Approval of Institutional Animal Care and Use (IACUC)

UNIVERSITI MALAYSIA KELANTAN

Fakulti Perubatan Veterinar

RUJ. KAMI *(Our Ref.)* :
TARIKH *(Date)* : UMK/FPV/ACUE/RES/001/2023
20 MARCH 2023

DR. GIGURUWA GAMAGE THILINI NISANSALA
Principal Investigator
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/RES/001/2023***
TITLE	***ELUCIDATION OF LEPTOSPIRA OCCURRENCE IN COMPANION DOGS IN KELANTAN***

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.

"ALLAH DITAATI, RAJA DISANJUNGI, RAKYAT DIKASIHI"
"MALAYSIA MADANI"
"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

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

UNIVERSITI MALAYSIA KELANTAN اونيۏرسيتي مليسيا كلنتن | Karung Berkunci *(Locked Bag)* 36, Pengkalan Chepa, 16100 Kota Bharu, KELANTAN, MALAYSIA | Tel : 609 771 7277 Fax : 609 771 7282

UNIVERSITI MALAYSIA KELANTAN

FACULTY OF VETERINARY MEDICINE UNIVERSITI MALAYSIA KELANTAN

Client Consent Form

Research Title: Elucidation of Leptospira occurrence in companion dogs in Kelantan

We would like to invite you to participate in a study to detect the presence of Leptospira in dogs.

In order to conduct this study, we would to seek for your consent to collect blood and urine samples that will be used for Leptospira culture, serology and molecular test. All sample collection will be done by an experienced registered veterinarian.

Every measure will be taken to ensure that the process of sample collection will be done as stress-free as possible with minimal or no risk to the dog(s).
If you decide to participate in this project, we are offering a free diagnostic workout for Leptospirosis in your dog worth RM 200. Further your dog will be benefited with deworming medication and tick control medication.
We shall ensure that all personal information obtained will be held confidential. We will inform you regarding the result for the entire test conducted as Leptospirosis is a zoonotic disease.
Thank you for your participation and helping us in this study.

I have voluntarily agreed to partake in this project. I understand that I can withdraw my consentat any time. I hereby give my consent for the participation in this project.

Owner's Name:_____Animal's name:_____

Case No: _____ Signature: _____

Email address: _____ Date: _____

Person to contact, if any problem arises: Dr. Thilini Nisansala (019-8909753) / Dr. Intan Noor Aina Binti Kamaruzaman (013-2243453) Dr. Mohammad Sabri Bin Abdul Rahman (013-6339874) Email address: thilini@umk.edu.my / intanaina@umk.edu.my / sabri.ar@umk.edu.my

A copy of Questionnaire on the Dogs Sampled

Elucidation of *Leptospira* occurrence in companion dogs in Kelantan

Case number: ……..... Date: ……

Hospital: ……..... Specimen number: ……...

Pet's Information

1. Dog's Name: ……

2. Dog's Age: ……

3. Dog's Breed: …….

4. Sex of the Dog: Male ☐ Female ☐ Entire ☐ Sterilized ☐

5. Place of residence: Urban area ☐ Rural area ☐

6. Housing of your Dog: Free roaming/outdoor ☐ Indoor ☐ Caged ☐

7. Are there any other pets in the home? Yes ☐ No ☐

If yes, please check all that apply and indicate the number of additional pets.

Dogs ……

Cats …….....

Other (Specify) ……

8. Dog's vaccination status: Yes ☐ No ☐

If yes, whether dog vaccinated against leptospirosis: Yes ☐ No ☐

If yes, number of vaccines received: ……..

Date of last vaccination against leptospirosis: ……..

9. Does your dog has any medical condition, Yes ☐ No ☐

If yes, what is the condition? ……

10. Is your dog receiving any medication currently? Yes ☐ No ☐

If yes, which medications? ……

11. Has your dog received antibiotics during the past six months? Yes ☐ No ☐

12. Does your dog come into any contact with rats? Yes ☐ No ☐

13. Does your dog drink water outside? Yes ☐ No ☐

If yes, where? …….. (puddles, ditches, lakes, sea etc.)

14. Does your dog have any recent outdoor/recreational activity? Yes ☐ No ☐

If yes, what is it? …….. (Outdoor walk, swimming in sea etc.)

15. Does your dog expose to flooding recently?

If yes, month and year of exposure ……

Samples obtained.

Blood ☐

Urine ☐

Voided ☐

Manual compression ☐

Cystocentesis ☐

Table of Demographic data of dogs sampled in Kelantan (*n* = 31)

No	Age (year)	Sex	Breed	Management	Vaccination Status	Place (District)
1.	1	Female	Chesapeake Bay	Indoor	Up to date	Bachok
2.	1	Female	Chesapeake Bay	Indoor	Up to date	Bachok
3.	1	Female	Chesapeake Bay	Outdoor	Not in list	Bachok
4.	<1	Male	Chesapeake Bay	Indoor	Not in list	Bachok
5.	11	Female	Belgian Malinois	Indoor	Up to date	Bachok
6.	11	Female	Schnauzer	Indoor	Not in list	Bachok
7.	<1	Male	Mongrel	Indoor	Not in list	Bachok
8.	11	Male	Poodle	Indoor	Not in list	Bachok
9.	11	Male	Labrador	Indoor	Not in list	Bachok
10.	6	Male	Labrador	Indoor	Not in list	Bachok
11.	2	Female	Mongrel	Outdoor	Up to date	Pasir Mas
12.	2	Female	Mongrel	Outdoor	Up to date	Pasir Mas
13.	1	Male	Mongrel	Outdoor	Up to date	Kota Bharu
14.	<1	Male	Mongrel	Indoor	Up to date	Kota Bharu
15.	<1	Female	Mongrel	Indoor	Up to date	Kota Bharu
16.	2	Female	Mongrel	Outdoor	Up to date	Kota Bharu
17.	2	Female	Mongrel	Outdoor	Up to date	Kota Bharu
18.	>1	Female	Mongrel	Outdoor	Not in list	Kota Bharu
19.	>1	Male	Poodle	Indoor	Up to date	Kuala Krai
20.	>1	Female	Poodle	Indoor	Up to date	Kuala Krai
21.	>1	Female	Poodle	Indoor	Up to date	Kuala Krai
22.	>1	Female	Poodle	Indoor	Up to date	Kuala Krai
23.	15	Male	Shih Tzu	Outdoor	Up to date	Bachok
24.	>1	Male	Mongrel	Outdoor	Not in list	Kota Bharu
25.	>1	Male	Mongrel	Outdoor	Not in list	Kota Bharu
26.	>1	Male	Mongrel	Outdoor	Not in list	Kota Bharu
27.	>1	Male	Mongrel	Outdoor	Not in list	Kota Bharu
28.	>1	Male	Mongrel	Outdoor	Not in list	Kota Bharu
29.	>1	Female	Mongrel	Outdoor	Not in list	Kota Bharu
30.	>1	Female	Mongrel	Outdoor	Not in list	Kota Bharu
31.	>1	Female	Mongrel	Outdoor	Not in list	Kota Bharu