Molecular Detection and Risk Factors of Leptospirosis Among Goats in Kelantan, Malaysia

By

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Faculty of Veterinary Medicine

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MOLECULAR DETECTION AND RISK FACTORS OF LEPTOSPIROSIS AMONG GOATS IN KELANTAN, MALAYSIA

ABSTRACT

This study investigates the molecular detection and risk factors of leptospirosis in goats in Kelantan, Malaysia. This study focuses on its zoonotic implications, economic impact on the livestock industry and the need for effective prevention strategies. Leptospirosis is caused by pathogenic *Leptospira* spp. It is a significant zoonotic disease that affects not just animals but humans as well. In livestock animals it can lead to productivity losses, abortions and even mortality. Despite its importance, limited research has been conducted on leptospirosis in goats in Malaysia, leaving gaps in understanding its epidemiology. A total of forty (n=40) blood samples from goats from 2 selected farms in Kelantan were analysed using polymerase chain reaction (PCR) to detect *Leptospira*-specific genes. The study also tried to assess potential risk factors such as age, sex, herd size and biosecurity practices. Findings of PCR analysis yielded zero detection rate. This could be due to factors such as small sample size, diagnostic sensitivity or the fact that all recruited animals were healthy and had no clinical signs related to leptospirosis. The findings also highlight the need for more extensive studies by using multiple diagnostic tools with multiple samples such as urine and tissue samples.

Keywords: Leptospirosis, goats, PCR, zoonotic, Malaysia

PENGESANAN MOLEKULAR DAN FAKTOR RISIKO LEPTOSPIROSIS DALAM KALANGAN KAMBING DI KELANTAN, MALAYSIA

ABSTRAK

Tesis ini mengkaji tentang pengesanan molekul dan faktor risiko leptospirosis kepada kambing di Kelantan, Malaysia. Kajian ini menumpu terhadap impak zoonosis, kesan ekonomi ke atas industri ternakan dan strategi pencegahan leptospirosis yang efektif. Leptospirosis disebabkan oleh bakteria *Leptospira* spp. Leptospirosis merupakan penyakit zoonosis penting yang menjejaskan haiwan dan manusia. Penyakit ini boleh mengakibatkan kesan negatif terhadap sistem pembiakan dan kematian dalam haiwan ternakan. Kini, penyelidikan mengenai leptospirosis dalam haiwan ternakan kambing di Malaysia adalah terhad. Hal ini telah menyebabkan kurangnya pemahaman terhadap epidemiologi penyakit ini. Sebanyak empat puluh (n=40) sampel darah kambing dari dua ladang terpilih di Kelantan telah dianalisis menggunakan tindak balas berantai polimerase (PCR) untuk mengesan gen khusus *Leptospira*. Kajian ini juga melihat kepada faktor risiko seperti umur, jantina, saiz kelompok ternakan dan amalan biosekuriti. Hasil analisis PCR menunjukkan kadar pengesanan adalah 0%. Hal ini mungkin disebabkan oleh faktor seperti saiz sampel yang kecil, sensitiviti diagnostik atau keadaan kesihatan haiwan yang diambil sebagai sampel tidak menunjukkan simptom klinikal yang berkait dengan leptospirosis. Penemuan ini juga menekankan keperluan untuk kajian yang lebih mendalam dengan menggunakan pelbagai alat diagnostik serta sampel yang berlainan seperti air kencing dan sampel tisu.

Kata kunci: Leptospirosis, kambing, PCR, zoonotic, Malaysia

CERTIFICATION

This is to certify that we have read this research paper entitled **'Molecular Detection and Risk Factors of Leptospirosis Among Goats in Kelantan, Malaysia'** by **Arissa Nabila Binti Mohd Rais** and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirements for the course DVT 55204 - Research Project.

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LIST OF ABBREVIATIONS

bp	Base pair
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
IACUC	Institutional Animal Care and Use Committee
MAT	Microscopic agglutination test
NFW	Nuclease- free water
PCR	Polymerase Chain Reaction
PBS	Phosphate-buffered saline
rRNA	Ribosomal RNA
TAE	Tris-acetate EDTA
UMK	Universiti Malaysia Kelantan
FPV	Faculty of Veterinary Medicine
CBC	Complete Blood Count
RBPT	Rose Bengal Plate Test

LIST OF SYMBOLS

°C	Degree celsius
%	Percentage
g	Gram
>	Greater than
≥	Greater than or equal to
<	Less than
ml	Mililitre
µL	Microlitre
µM	Micromolar
n	Number of individuals in the sample size
g	Times gravity
™	Trademark sign
®	Registered trademark sign
V	Volt

CHAPTER 1

INTRODUCTION

Leptospirosis is a disease caused by pathogenic *Leptospira* spp. Leptospiral infection is endemic in Malaysia and predominantly occurs in a subclinical form (Darus *et al.*, 2013). It can affect a wide range of hosts such as cattle, sheep, goats, dogs, cats and even humans. It is a zoonotic disease and this disease threatens public health and the economy of ruminant farms thus becoming one of the notifiable diseases to the authority (DVS, 2011). The zoonotic risk arises when infected animals transmit the disease to farm workers and veterinarians through direct contact with urine and bodily fluids or indirectly through contaminated water and soil.

Research on leptospiral infection in Malaysian goats is limited. Existing studies, like those in Kelantan, have focused on the link between leptospirosis and flooding (Sabri *et al.*, 2019) and (Rahman *et al.*, 2021). Research on leptospirosis in goats is crucial for safeguarding public health and the livestock industry. Understanding infection rates, transmission pathways and risk factors enables the development of effective control measures, reducing economic losses, improving animal health and preventing zoonotic transmission to humans especially farm workers and veterinarians.

1.1 Research Problem Statement

The zoonotic risk of leptospirosis arises when diseased goats transmit the infection to farm workers and veterinarians through direct contact with urine and bodily fluids or indirectly through contaminated water and soil. Despite the known risks, research on leptospiral infection in goats in Malaysia is limited where there are two existing studies in the Kelantan region primarily focused on the association between leptospirosis and massive flooding (Sabri *et al.*, 2019) and (Rahman *et al.*, 2021). Due to the environmental and occupational exposure risks, understanding the occurrence and specific *Leptospira* species circulating among goats is crucial for developing effective control measures. Therefore, this study aims to detect *Leptospira* spp. together with its level of occurrence and its associated risk factors.

1.2 Research Question

I. What is the level of occurrence of leptospirosis in goats in the two selected districts in Kelantan, Malaysia?

II. What are the most prevalent *Leptospira* species detected among goats in Kelantan, Malaysia?

III. What are the risk factors associated with leptospirosis in goats in Kelantan, Malaysia?

1.3 Research Hypothesis

I. There is a low to moderate level of leptospirosis among goats in Kelantan, Malaysia.

II. The most prevalent *Leptospira* species detected among goats in Kelantan Malaysia is *L. interrogans*.

III. There is an association between the factors of age, sex, breed, size of herd, educational level of the farmer, contacting an institution for technical support and biosecurity with leptospirosis in goats in Kelantan, Malaysia.

1.4 Research Objectives

I. To determine the level of occurrence of *Leptospira* spp. in goats in Kelantan, Malaysia with molecular detection method.

II. To identify the most prevalent *Leptospira* species among goats in Kelantan, Malaysia.

III. To determine the association between risk factors and the occurrence of leptospirosis among goats in Kelantan, Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of *Leptospira* spp.

Leptospira spp. are spiral-shaped, Gram-negative bacteria belonging to the genus *Leptospira* within the family Leptospiraceae. They are classified into pathogenic, intermediate and saprophytic species based on their biological characteristics and molecular phylogeny (Fouts *et al.,* 2016). Leptospires are motile, aerobic organisms with unique hook-shaped ends that allow them to move efficiently through water and penetrate host tissues.

Pathogenic *Leptospira* species are the causative agents of leptospirosis. It is a zoonotic disease that affects humans and a wide range of domestic and wild animals. These species include *L. interrogans, L. kirschneri, L. noguchii* and *L. santarosai* among others. Each species is further divided into serovars based on antigenic differences in their lipopolysaccharide (LPS) surface antigens (Adler & Moctezuma, 2010).

Saprophytic *Leptospira* species like *Leptospira biflexa* are naturally found organisms in soil and water environments. They play a role in maintaining the ecological balance of their habitats and are not known to cause leptospirosis (Mohammed *et al.,* 2011).

The transmission of pathogenic *Leptospira* spp. occurs through direct or indirect contact with the urine or tissues of infected animals, contaminated water or soil. Rodents and wild mammals serve as the primary reservoir hosts and many other domestic animals including dogs and livestock can also harbour and shed the bacteria as carriers (Yadeta *et al.,* 2016).

2.2 Caprine leptospirosis

Most leptospirosis in goats is asymptomatic but they can still contribute to the spread of *Leptospira* by excreting the bacteria in their urine, thereby playing a role in the epidemiology of the disease. Caprine leptospirosis occurs globally but is particularly prevalent in tropical and subtropical regions (Hajikolaei *et al.,* 2022). This is because they tend to survive longer in warm and humid environments.

Goats can become infected with *Leptospira* through direct or indirect contact with urine or tissues of infected animals (Martins & Lilenbaum, 2014). Rodents, particularly rats, are considered primary reservoir hosts for *Leptospira* and play a significant role in disease transmission to goats through contamination of the environment with their urine. In goats, the infection leads to abortions, infertility, or other reproductive issues resulting in considerable animal and economic losses (Lima *et al.*, 2017).

2.3 Diagnosis of leptospirosis

According to Pinto *et al.* (2022) leptospirosis is diagnosed through various methods including the detection of *Leptospir*a in blood and urine using direct dark-field microscopy (DFM), isolation of the bacteria through culture, identification of genomic DNA via molecular techniques such as polymerase chain reaction (PCR) and detection of antibodies using serological tests like the microscopic agglutination test (MAT).

The PCR-based assays are becoming more popular for detecting leptospires in animal tissues and body fluids due to their reputed sensitivity and ability to provide early diagnosis (WOAH, 2021). Polymerase chain reaction is a highly sensitive and specific molecular technique used for diagnosing leptospirosis by amplifying the DNA of *Leptospira*.

Microscopic agglutination test is considered the gold standard for diagnosing leptospirosis. This serovar or serogroup-specific test identifies leptospiral serogroups and necessitates a panel of live pathogenic *Leptospira* strains as reference (Pinto *et. al*, 2022). For optimal sensitivity, usage of MAT should utilise antigens that represent all serogroups known to exist in the region where the animals are located.

2.4 Treatment and prevention of caprine leptospirosis

Treatment and prevention strategies for leptospirosis in goats are crucial for managing the disease and minimising its impact on goat health and productivity. Treatment primarily involves early administration of antibiotics such as penicillin and doxycycline together with supportive intravenous fluid administration (Shaheena, 2020). Additionally, infected goats should be isolated from healthy animals to prevent disease spread within the herd and quarantine measures should be implemented for newly introduced animals to prevent disease introduction.

To prevent and reduce the spread of leptospirosis, good animal management practices can be helpful (CDC, 2024). Vaccination against leptospirosis is recommended to prevent infection and reduce transmission within the herd. However, according to the Department of Veterinary Services of Malaysia (2024), no leptospirosis vaccination has been approved for the usage in goats.

2.5 Risk factors of leptospirosis

Ruminants are particularly susceptible to the disease and understanding the risk factors associated with leptospirosis is crucial for developing effective prevention and control strategies. A study in small ruminants of north-eastern Colombia revealed that there was no correlation between *Leptospira* spp. seroprevalence and variables such as sex, water source, presence of other animals and weaning time. There was also no statistical correlation for variables such as the absence of disinfection, the presence of faeces in the perimeter, the lack of rodent control, and animal production on land. However, identified risk factors were found to be the absence of housing system and lack of quarantine measures for new animals (Guzman-Barragan *et al.,* 2022). A study in goats of Northeast Brazil stated that four variables were identified as factors associated with *Leptospira* sp. seroprevalence which are the sex of the animal (female), educational level of the farmer, engagement with an institution for technical support and presence of an irrigation area on the property (Viana *et al.,* 2022). According to a study of leptospirosis in ruminants done in Kelantan before, flood could also possibly be a major risk in distributing and transmitting the disease to goats (Sabri *et al.,* 2019) and (Rahman *et al.,* 2021).

Some factors associated with leptospirosis risk factors but not limited to goats also had shown that in cattle, the likelihood of older cows shedding *Leptospira* decreased by 18% for each additional year of age. Large herds were more likely to harbour shedders than small herds. As for most pathogens, several studies have shown that large herd size was associated with a higher risk of *Leptospira* transmission in cattle due to more frequent contact between infectious and susceptible animals (Yupiana *et al.,* 2020). Maybe this could be the same concept for goat herds as well.

2.6 Economical impact of leptospirosis

Leptospirosis poses a significant economic burden globally. This infection is considered a leading cause of economic losses in livestock, as it contributes to reproductive issues and poses a significant agri-food vulnerability due to its impact on livestock productivity (López-Robles *et al.*, 2021). Leptospirosis results in economic losses through oestrus repetition or the birth of weak offsprings (Orjuela *et al.*, 2022). These delays breeding schedules and increases the time between interbirth intervals, reducing the overall number of offsprings produced over the animal's lifetime. Weak offsprings may require additional veterinary care, special feeding or may not survive, leading to direct losses in potential income from livestock sales or dairy production.

As leptospirosis is zoonotic, this disease affects humans as well and can lead to substantial healthcare costs, including hospitalization, diagnostic tests and treatments. According to Saizan *et al.* (2019), the state of Kelantan could gain between US$101.70 million as leisure rate and US$315 million of wage rate by preventing leptospirosis. This can also be interpreted as the potential economic loss if leptospirosis is not addressed. The economic impact extends to the loss of income due to loss of human productivity, particularly in individuals living in resource-poor settings in tropical and subtropical regions (Agampodi *et al.*, 2023).

2.7 Significance of expected research findings

This research study can help in understanding that leptospirosis poses a substantial public health risk especially in tropical countries where environmental conditions favour *Leptospira* survivability. Goats can serve as reservoirs and thus transmit the pathogen to humans directly or through contaminated water and soil. Identifying risk factors associated with leptospirosis in goats can aid in prevention strategies towards leptospirosis as economically, leptospirosis can lead to reproductive issues, decreased milk production and overall poor health in goats which can significantly impact the livelihoods of farmers who rely on goat farming. This research might also help further research to consider implementing *Leptospira* vaccination in Malaysia where this vaccination is not commonly used in livestock.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Ethical Approval

Ethical approvals for using animals in the current study was obtained from the Animal Ethics Committee, Faculty of Veterinary Medicine of Universiti Malaysia Kelantan under the approval code of UMK/FPV/ACUE/FYP/007/2024 (Appendix A).

3.2 Study Area and Target Population

The study area involved two goat farms in Kelantan, Malaysia. There are eleven districts in Kelantan. One farm in Kota Bharu and another farm in Bachok districts were chosen for this current study.

3.3 Acquiring Consent and Subject Medical History

Consent was given both verbally and written by the owner. History was taken verbally and recorded on paper.

3.4 Sample Collection

A total of 40 blood samples were collected from goats in Kelantan, Malaysia in July 2024. The blood was aseptically drawn from the jugular vein, with each goat providing one sample stored in an Ethylenediaminetetraacetic acid (EDTA) tube and another in a plain tube. Both tubes were appropriately labelled and the samples were kept chilled in a cold box packed with ice during transportation. After arriving at the laboratory, samples were then stored in a chiller at 4°C.

Table 3.4.1: Samples collected according to districts in Kelantan.

No.	District	No. of samples collected
1	Kota Bharu	23
2	Bachok	17
	Total	40

3.5 DNA Extraction

DNA extraction from blood samples was performed using the Geneaid gSYNC™ (Geneaid Biotech Ltd., Taiwan R.O.C.) commercial DNA extraction kit following the manufacturer's protocol. All necessary consumables, including 1.5 ml microcentrifuge tubes, micropipette tips and PCR tubes, were sterilised by autoclaving before the extraction process. The EDTA tubes were removed from storage and left to thaw at room temperature for 30 minutes before starting the extraction.

To begin, 200 µl of each blood sample was transferred into a 1.5 ml microcentrifuge tube. Then, 20 µl of proteinase K was added and mixed by pipetting. The samples were incubated at 60°C for 10 minutes.

For cell lysis, 200 µl of GSB buffer was added to the incubated samples which were then vigorously shaken using a vortex mixer. After mixing, the samples were incubated again at 60°C for 10 minutes. During this time, 200 µl of Elution Buffer per sample was placed into 1.5 ml microcentrifuge tubes and heated to 60°C for the final phase of the DNA extraction. GS columns were prepared by placing each into a 2 ml collection tube.

For DNA binding, 200 µl of absolute ethanol was added to the sample lysate which was then vigorously shaken for 10 seconds. The mixture was transferred into the prepared GS column

and centrifuged at 14,000 x g for 1 minute. The flow-through in the 2 ml collection tube was discarded and the GS column was placed in a new 2 ml collection tube.

During the washing step, 400 µl of W1 buffer was added to the GS column, followed by centrifugation at 14,000 x g for 30 seconds. The flow-through was discarded, and the column was reinserted into the collection tube. Then, 600 µl of wash buffer was added and the column was centrifuged again for 30 seconds at 14,000 x g. After discarding the flow-through, the GS column was centrifuged for an additional 3 minutes at 14,000 x g to ensure dryness.

For DNA elution, the dried GS column was transferred to a clean 1.5 ml microcentrifuge tube. Next, 100 µl of the pre-heated Elution Buffer was applied to the centre of the column and it was left to stand for 5 minutes to allow absorption. The column was then centrifuged for 30 seconds at 14,000 x g to elute the purified DNA. The extracted DNA samples were stored in a cryobox at -20°C.

3.6 Polymerase Chain Reaction (PCR)

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

A 25 µl PCR reaction mixture was prepared, comprising 12.5 µl of PCR master mix (e.g., GoTaq), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer and 5.5 µl of nuclease-free water. This mixture was scaled up by a factor of 13 for the first batch of PCR reactions, accounting for 10 samples, 1 positive control, 1 negative control and 1 extra unit to account for potential pipetting errors (Table 3.6.1). The PCR components were combined in a 1.5 ml microcentrifuge tube. Then, 20 µl of this master mix was aliquoted into 12 PCR tubes. For sample testing, 5 µl of each sample DNA template was added to 10 of the tubes. The remaining tubes were prepared with 5 µl of *Leptospira* spp. positive control and 5 µl of nuclease-free water as the negative control, respectively.

Table 3.6.1: Calculation of first batch of components required for PCR

PCR Components	1 unit	13 units
Master Mix	12.5	162.5
Forward Primer	1	13
Reverse Primer	1	13
Nuclease-Free Water (NFW)	5.5	71.5
Sample DNA	5	-
TOTAL	**25**	-

The PCR amplification was carried out using a thermal cycler (Bio-Rad Laboratories, Inc., Unites States) The program began with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60.4°C for 30 seconds, and extension at 72°C for 60 seconds. A final extension step was performed at 72°C for 5 minutes.

For the second batch of PCR, the reaction mixture was scaled up by a factor of 34 to accommodate 30 sample reactions, 1 positive control, 1 negative control, and 2 extra units (Table 3.6.2). The prepared PCR mixture was divided into 32 PCR tubes, with 20 µl of the mix added to each tube. Then, 5 µl of DNA template was added to 30 tubes for the samples. As before, 5 µl of positive control and 5 µl of nuclease-free water were added to the remaining two tubes as positive and negative controls, respectively.

Table 3.6.2: Calculation of second batch 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
Nuclease-Free Water (NFW)	5.5	187
Sample DNA	5	-
TOTAL	**25**	-

3.7 Gel Electrophoresis

The amplified PCR products were analysed using agarose gel electrophoresis. To prepare the gel, 1.2 g of agarose powder was measured and mixed with 80 ml of 1× TAE buffer in a Schott bottle to create a 1.5% (w/v) agarose solution. The mixture was microwaved for 1 minute and 40

seconds until the agarose was completely dissolved. Once dissolved, 1 µl of Midori Green dye was added to the hot agarose solution for visualisation.

For the first batch of PCR products, a 20-well comb was placed in a gel tray, and the prepared agarose solution was carefully poured into the tray. The gel was allowed to solidify at room temperature for 30 minutes. After the gel had set, the comb was gently removed, and the gel was placed in an electrophoresis tank filled with a 1× TAE buffer.

A 100 bp DNA ladder (5 µl) was loaded into the first well, while 10 µl of PCR products were loaded into the remaining wells each. The positive and negative controls were added to the last two wells. Electrophoresis was performed at 100 V and 400 mA for 45 minutes. After the run, the gel was removed from the tank and visualised using the ImageQuant™ LAS 500 imager (GE Healthcare Life Sciences, United States) which revealed DNA bands at approximately 330 bp.

For the second batch of PCR products, the same steps were repeated. However, to accommodate 30 amplified products, both a 30-well comb and a 20-well comb were used. This allowed for efficient loading and electrophoresis of all samples. On the 30-well comb, 5 µl 100 bp DNA ladder was loaded into the first well, followed by the loading of 20 PCR products into the subsequent wells. While on the 20-well comb, 5 µl 100 bp DNA ladder was loaded into the first well, followed by the loading of another 10 PCR products into the subsequent wells. Then, positive and negative controls were added to the last two wells.

3.8 Health screening

Two additional diagnostic tests, the Rose Bengal Plate Test (RBPT) and blood profiling, were conducted as part of a comprehensive health screening. The RBPT was performed on all 40 goat blood samples to screen for brucellosis, providing added value since brucellosis also impacts the reproductive system. For the test, the serum samples and Rose Bengal antigen were first brought to room temperature. A drop of serum was then placed on a clean glass slide followed by an equal volume of Rose Bengal antigen added adjacent to it. Using a mixing stick, the serum and antigen were thoroughly mixed to form a homogenous mixture. The mixture was left to react for approximately 4 minutes at room temperature while being gently rotated in a circular motion. The reaction was then observed against a white background. Agglutination indicated a positive result,

signifying the presence of antibodies against *Brucella*, while a smooth, homogenous mixture without clumping was recorded as a negative result.

For blood profiling, the samples were submitted to the Clinical Pathology Laboratory for detailed analysis and results.

CHAPTER 4

FINDINGS AND DISCUSSION

4.1 Demographic Data of Goats

Table 4.1.1 shows the demographic data of animals in the study (details of the data can refer to Appendix B). This table summarizes data on goat samples categorized by age, sex, management practices and health status along with their respective percentages. Out of 40 goat samples, 10% (4 goats) are less than 1 year old while 90% (36 goats) are over 1 year old. 60% (24 goats) are male, and 40% (16 goats) are female. 57.5% (23 goats) are reared under intensive management while 42.5% (17 goats) are under semi-intensive management. Lastly, 95% (38 goats) are healthy and 5% (2 goats) have other diseases which was flu and diarrhoea.

Table 4.1.1: Demographic data of animals in the study

	No. of goat samples	Percentage (%)
Age		
<1 year old	4	10
>1 year old	36	90
Sex		
Male	24	60
Female	16	40
Management		
Intensive	23	57.5
Semi-intensive	17	42.5
Health status		
Healthy	38	95
Other diseases	2	5

4.2 Result of PCR

The amplification of the 16S rRNA gene for *Leptospira* spp. revealed that all samples tested negative by PCR. As illustrated in Figures 4.2.1 and 4.2.2, no bands were observed in the sample lanes at the expected position corresponding to the 330 bp band for leptospiral DNA, as seen in the positive control. The absence of bands in the sample lanes confirms that none of the 40 samples tested positive for the 16S rRNA gene of Leptospira spp. The presence of a band in the positive control lane, corresponding to 330 bp, validates the PCR procedure and confirms the successful detection of *Leptospira* spp. DNA.

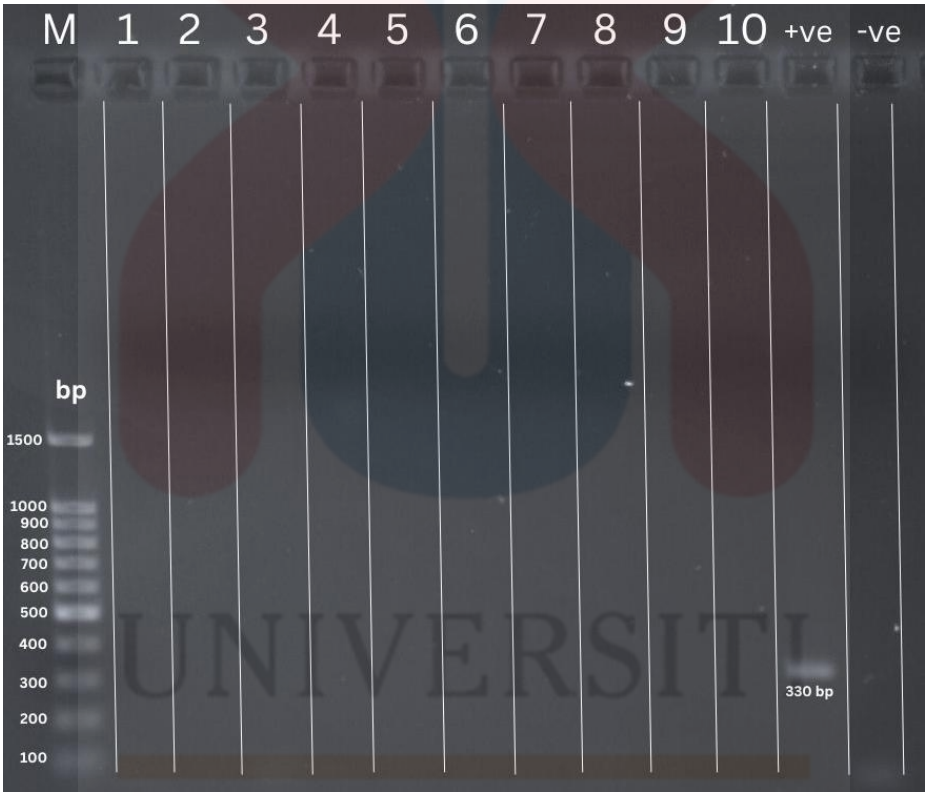

Figure 4.2.1: Gel electrophoresis image of the initial batch of PCR, demonstrating the amplification of the 16S rRNA gene from goat blood samples. 'M' indicated the 100 bp DNA ladder, lanes '1-10' displayed the PCR products from the samples, '+ve' served as the positive control and'-ve' represented the negative control.

Figure 4.2.2: Gel electrophoresis image for the second batch of PCR illustrating the amplification of the 16S rRNA gene from goat blood samples. 'M' indicated the 100 bp DNA ladder, lanes '1-30' showed the PCR products from the samples, '+ve' served as the positive control and '-ve' represented the negative control.

Fortunately, none of the goats tested positive as evidenced by the absence of agglutination when their serum was mixed with the RBPT reagent. To further evaluate the health status of the herd, blood from 10 randomly selected goats was sent for complete blood count (CBC) and serum biochemistry analysis. The CBC provided data into the goats' haematological profiles including red and white blood cell counts, haemoglobin levels and haematocrit values while the serum biochemistry assessed parameters of kidney function through blood urea nitrogen and creatinine value.

Table 4.2.3: Parameters analysed for CBC and serum biochemistry of 10 randomly selected goats.

Parameters	High	Normal	Low
Haemogram			
WBC	9	1	0
LYM	10	0	0
MON	7	3	0
GRA	9	1	0
RBC	10	0	0
HGB	0	0	10
HCT	0	6	4
Biochemistry			
BUN	9	1	0
CREA	10	0	0

4.3 Discussion

The PCR analysis for detecting *Leptospira* spp. in goat samples from Kelantan yielded zero detection rate with no positive results observed. Several factors could have contributed to this outcome which highlights the limitations of the current study and the complexity of diagnosing leptospirosis in caprine populations.

The sample size in this study was limited to 40 goats which is insufficient for producing representative results. Kelantan has an estimated goat population of 32,596 (DVS, 2023). Using an appropriate sample size calculation, a sample size of 380 would be required for statistically significant findings. The small sample size in this study may not have adequately captured the diversity and prevalence of *Leptospira* spp. in the population which leads to the lack of positive detections. A larger sample size is necessary to increase the probability of identifying infected individuals and obtaining a more accurate detection rate.

Goats were sampled from only two farms of two different districts in Kelantan which may not reflect the broader goat population across this state. The limited geographic representation may have contributed to the 0% detection rate as different farms or districts may have varying environmental conditions and risk factors for leptospirosis.

Most of the goats in this study were raised intensively on raised floors, which reduces their exposure to potential sources of leptospiral infection such as muddy soil or stagnant water (Goarant, 2016). This management style could have contributed to the 0% detection rate. Even the goats that were semi-intensive, once they were back in the pen, they were raised on raised floors. Future studies should include free-roaming goats which are more likely to be exposed to environmental risk factors associated with leptospirosis.

The sampling took place during the hot season, which also might lead to the 0% detection rate. Even the findings by Sabri *et al.* (2019), reported a low detection rate despite using a larger sample size and considering flood as a risk factor, with only 4 positive results out of 635 blood samples. Environmental factors such as flooding, which increases exposure to contaminated water are critical risk factors for leptospirosis. The absence of these conditions during sampling may explain the lack of positive results.

This study relied exclusively on blood samples for PCR analysis which may not be sufficient for detecting *Leptospira* spp. The bacterium can be present in various body fluids including urine and milk and isolation of leptospires in internal organs such as liver, lung, brain and kidney can give a definitive diagnosis of this disease in the acute clinical stage or even in chronic infection stage (WOAH, 2021). The absence of these other types of samples limits the scope of the study and may have contributed to the negative findings.

The microscopic agglutination test (MAT) has traditionally been regarded as the gold standard for diagnosing leptospirosis (Hartskeerl & Smythe, 2015). From a study done by Hajikolaei *et al.* (2022), out of 21 sheep and 23 goats that tested positive using the microscopic agglutination test (MAT), none were found to be positive through polymerase chain reaction (PCR). But on the other hand, all samples that were positive in PCR lacked detectable antibodies to *Leptospira interrogans* in MAT. This difference can be explained by the fact that the two diagnostic methods detect different aspects of the infection and are effective at different stages of the disease. Microscopic agglutination test is commonly used to determine antibody titres for diagnosing leptospirosis. A fourfold or greater increase in antibody titres between acute and convalescent serum samples indicates a recent leptospiral infection (Chirathaworn *et al.*, 2014). Polymerase chain reaction, on the other hand, detects the presence of bacterial DNA indicating an active infection. Once the infection resolves or bacterial load decreases below the detection threshold, PCR results may turn negative. Thus, the PCR-negative results in MAT-positive animals suggests that these animals may have been exposed to *L. interrogans* earlier but were no longer actively infected at the time of sampling. Similarly, the PCR-positive but MAT-negative samples could indicate recent infections where the immune response had not yet developed sufficiently to be detected by MAT. However, in a study done by Philip *et al.* (2020), eleven human patients were found to be positive in both PCR and MAT in the range of 1-8 days post-onset of symptoms. This incident can happen when the timing of antibody production overlaps with the period when leptospires are still present in the blood (Day, 2022). This highlights the importance of using complementary diagnostic methods to capture different stages of infection for a more comprehensive understanding of leptospirosis epidemiology.

This study hypothesised that several factors including age, sex, breed, herd size, farmer's educational level, engagement with institutions for technical support and biosecurity practices may

be associated with leptospirosis in goats in Kelantan, Malaysia. However, due negative findings, the study could not determine whether the hypothesis could be accepted or rejected as statistical analysis was not feasible. If any positive results had been obtained through PCR testing, the corresponding samples would have been subjected to Sanger sequencing to identify the *Leptospira* species. Unfortunately, since all samples tested negative, this aspect of the study could not be pursued. As a result, the study was unable to determine the most prevalent *Leptospira* species among goats in Kelantan, nor could it identify any risk factors associated with the infection. These research questions remain unanswered emphasising the need for a more extensive study design. Future research should aim to include a larger sample size, incorporate multiple sample types and make use of complementary diagnostic techniques such as MAT.

Most of the goats (38 goats) were healthy and only two showed clinical symptoms. However, the symptoms were limited to diarrhoea and flu. History of typical signs of leptospirosis such as reproductive losses were also not reported by the farmer. Though goat infections are usually asymptomatic, the inclusion of mostly healthy goats may have contributed to the negative results in this study. Future research including goats with relevant symptoms of leptospirosis might produce different findings.

The Rose Bengal Plate Test (RBPT) was conducted as part of the health screening for all goats in the study. The results showed that all goats tested negative for brucellosis which indicates the absence of this bacterial infection in the herd. This negative outcome suggests that the goats were free from *Brucella* spp. infections which could have significantly impact reproductive health and general well-being. The Rose Bengal test can detect both agglutinating and non-agglutinating antibodies and avoids false negatives caused by the prozone effect which makes it highly sensitive for identifying *Brucella* infections, no matter the disease stage (Ruiz-Mesa *et al.*, 2005). According to a study by Legesse *et al*. (2023), the sensitivity of RBPT was 100% (95% CI) in goats while the specificity was 99.28% (95% CI). Another study by Ipola *et al.* (2018), RBPT showed a sensitivity of 100% and a specificity of 91%. The high sensitivity and specificity of the RBPT, as demonstrated in previous studies, confirm that the negative results can be trusted, indicating that brucellosis was not present in the herd at the time of testing. Thus, the health screening successfully confirmed the absence of this bacterial infection, supporting the herd's health status which were all generally healthy at the time of sampling.

Nine out of ten goats showed an elevation in white blood cell (WBC) counts suggesting the presence of an underlying infection or inflammation. This finding indicates that the goats may be responding to a bacterial, viral, parasitic or even possibly a non-infectious inflammatory process. All 10 goats exhibited an increase in lymphocyte counts which may point to a chronic infection or a prolonged immune response. Chronic infections often stimulate lymphocyte proliferation as part of the adaptive immune response. Seven goats have higher than normal amounts of monocyte levels indicating a response to chronic inflammation or tissue damage, which might align with the elevated WBC counts. These findings do match the findings by a study where total leucocyte count (TLC) values were comparatively higher in leptospiral seropositive goats than in seronegative ones (Vihol *et al.*, 2016).

Interestingly, all the blood samples showed an increase in red blood cell (RBC) counts. While this might initially suggest polycythaemia, it is more likely a response to dehydration, as dehydration can concentrate the blood, artificially increasing RBC levels. All of the goats had a low haemoglobin amount. Six out of ten goats had a low haematocrit amount. This could indicate that the goats were anaemic. In a study, a significant decrease in packed cell volume (PCV), haemoglobin (Hb) and mean corpuscular haemoglobin concentration (MCHC) values was observed in seropositive goats (Vihol *et al.*, 2016). Though the RBC counts were high, the anaemia could be masked due to dehydration. This assumption is further supported by elevated blood urea nitrogen (BUN) and creatinine levels in the goats. Dehydration reduces renal perfusion leading to decreased clearance of these waste products which results in their accumulation in the blood. The above biochemical result differs from the findings by Vihol *et al.* (2016) whereby they found that biochemical tests did not show signs of kidney damage caprine leptospirosis. This is likely because kidneys can keep working normally for a long time due to their extra tissue until they reach a critical point where they can no longer recover.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, the 0% detection rate in this study can be attributed to multiple factors which are the small sample size, the use of blood samples alone and reliance solely on PCR for diagnosis. These limitations highlight the importance of a stronger study design to determine the occurrence of *Leptospira* spp. and the associated risk factors among goats in Kelantan, Malaysia. Future study should aim to overcome these limitations to provide a better understanding of leptospirosis in caprine populations in Kelantan.

5.2 Recommendations

Future research should address the limitations of this study by having a larger sample size to ensure statistically significant findings and better representation of the goat population. It should include goats managed under different systems such as free-roaming goats. This might provide insights into the impact of management practices on leptospirosis risk. To add on, future studies should incorporate a variety of samples including urine, milk and tissues from organs like the liver and kidneys to improve diagnostic sensitivity and capture different stages of infection. Seasonal and geographic variations should also be considered with sampling conducted during various seasons and in more districts to account for environmental and regional differences. The inclusion of goats that show clinical manifestations of leptospirosis might increase the likelihood of positive result. Lastly, studies should employ complementary diagnostic methods such as combining PCR with MAT as it can help detect both active and past infections.

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APPENDIX

Appendix A: A copy of Approval from the Institutional Animal Care and Use Committee (IACUC) of Universiti Malaysia Kelantan.

UNIVERSITI MALAYSIA KELANTAN
Kampus Kota, 16100 Kota Bharu, Kelantan, Malaysia.
www.umk.edu.my Tel : 09-7717000/7281

UNIVERSITI MALAYSIA KELANTAN

FAKULTI PERUBATAN VETERINAR
Faculty of Veterinary Medicine

Ruj. Kami (*Our Ref.*) : UMK/FPV/ACUE/FYP/007/2024
Tarikh (*Date*) : 11th August 2024

DR. MOHAMMAD SABRI BIN ABDUL RAHMAN
Co Supervisor
Faculty of Veterinary Medicine
University 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, University Malaysia Kelantan has approved. Please refer the table below for approval code:

APPROVAL CODE	*UMK/FPV/ACUE/FYP/007/2024*
TITLE	MOLECULAR DETECTION AND RISK FACTORS OF LEPTOSPIROSIS AMONG GOATS IN KELANTAN, MALAYSIA
NAME OF STUDENT	ARISSA NABILA BINTI MOHD RAIS

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.

"ISLAM MEMBIMBING, RAJA MEMIMPIN, NEGERI DIBERKATI"
"MALAYSIA MADANI"
"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

(DR. MOHAMMED DAUDA GONI)
Chairman
Institutional Animal Care and Use Committee
Faculty of Veterinary Medicine

Appendix B: Table of Demographic Data of Dogs Samples in Kelantan (n=40)

No.	**Goat ID**	**Sex**	**Age (year)**	**Management**	**Place (District)**	Health status
1.	N1	M	>1	Intensive	Kota Bharu	Healthy
2.	N2	M	>1	Intensive	Kota Bharu	Healthy
3.	N3	M	>1	Intensive	Kota Bharu	Healthy
4.	N4	M	>1	Intensive	Kota Bharu	Healthy
5.	N5	M	>1	Intensive	Kota Bharu	Healthy
6.	N6	M	<1	Intensive	Kota Bharu	Healthy
7.	N7	M	>1	Intensive	Kota Bharu	Healthy
8.	N8	M	>1	Intensive	Kota Bharu	Healthy
9.	N9	M	>1	Intensive	Kota Bharu	Healthy
10.	N10	M	<1	Intensive	Kota Bharu	Healthy
11.	N11	M	>1	Intensive	Kota Bharu	Healthy
12.	N12	M	>1	Intensive	Kota Bharu	Healthy
13.	N13	F	>1	Intensive	Kota Bharu	Healthy
14.	N14	F	>1	Intensive	Kota Bharu	Healthy
15.	N15	F	>1	Intensive	Kota Bharu	Healthy
16.	N16	F	>1	Intensive	Kota Bharu	Healthy
17.	N17	F	>1	Intensive	Kota Bharu	Healthy
18.	N18	F	>1	Intensive	Kota Bharu	Healthy
19.	N19	F	>1	Intensive	Kota Bharu	Healthy
20.	N20	F	>1	Intensive	Kota Bharu	Healthy
21.	N21	F	>1	Intensive	Kota Bharu	Diarrhoea
22.	N22	F	>1	Intensive	Kota Bharu	Healthy

23.	N23	F	>1	Intensive	Kota Bharu	Flu
24.	D1	F	>1	Semi-intensive	Bachok	Healthy
25.	D2	M	<1	Semi-intensive	Bachok	Healthy
26.	D3	F	>1	Semi-intensive	Bachok	Healthy
27.	D4	M	<1	Semi-intensive	Bachok	Healthy
28.	D5	F	>1	Semi-intensive	Bachok	Healthy
29.	D6	M	>1	Semi-intensive	Bachok	Healthy
30.	D7	F	>1	Semi-intensive	Bachok	Healthy
31.	D8	M	>1	Semi-intensive	Bachok	Healthy
32.	D9	M	>1	Semi-intensive	Bachok	Healthy
33.	D10	M	>1	Semi-intensive	Bachok	Healthy
34.	D11	M	>1	Semi-intensive	Bachok	Healthy
35.	D12	M	>1	Semi-intensive	Bachok	Healthy
36.	D13	M	>1	Semi-intensive	Bachok	Healthy
37.	D14	M	>1	Semi-intensive	Bachok	Healthy
38.	D15	M	>1	Semi-intensive	Bachok	Healthy
39.	D16	M	>1	Semi-intensive	Bachok	Healthy

40.	D17	F	>1	Semi-intensive	Bachok	Healthy