PREVALENCE OF LEPTOSPIROSIS AMONG CATS IN KELANTAN

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PREVALENCE OF LEPTOSPIROSIS AMONG CATS IN KELANTAN

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PREVALENCE OF LEPTOSPIROSIS AMONG CATS IN KELANTAN

ABSTRACT

Leptospirosis is an important zoonotic disease found in tropical countries including Malaysia which infects both humans and domestic animals. It is caused by pathogenic *Leptospira* spp. which causes subclinical infection and shedding of the pathogen through urine. This prompted further research to evaluate the zoonotic implications of feline leptospirosis in cats exposed to environmental risk factors in Kelantan. Hence, this study aims to determine the prevalence of leptospirosis and infecting *Leptospira* serovars among cats in Kelantan. A total of 30 blood samples (whole blood – 30 and serum – 26) were collected from cats presenting to selected veterinary clinics in Kelantan. Serum samples were tested with Microscopic Agglutination Test (MAT) and whole blood samples were subjected to Polymerase Chain Reaction (PCR). Based on the results, all of the cats were tested negative by PCR. Four cats (15.38.%) were tested positive for MAT with the cut-off antibody titre of ≥1:100. Of the 04 MAT positives, 03 were positive for serovar Canicola with a titre of 1:100 and 01 cat had a titre of 1:200 for serovar Bataviae. The detection of *Leptospira* specific antibodies within the study cohort holds significant implications for both public and animal health. The positive serological results pertaining to *Leptospira* are pivotal in contributing vital insights into local disease epidemiology, signalling a potential elevated risk of feline leptospirosis. These findings serve as a foundational resource for developing preventative strategies against feline leptospirosis in Kelantan, Malaysia. In conclusion, there were 15.38% seroprevalence of leptospirosis among cats that were enrolled for the study in Kelantan.

Keywords: Leptospirosis, Feline, MAT, PCR

PREVALENS LEPTOSPIROSIS DI KALANGAN KUCING DI KELANTAN

ABSTRAK

Leptospirosis merupakan penyakit zoonosis penting di negara tropikal termasuk Malaysia yang menjangkiti manusia dan haiwan peliharaan. Penyakit ini disebabkan oleh patogen *Leptospira* spp. yang menyebabkan jangkitan subklinikal pada kucing yang membawa kepada pathogen terdapat dalam air kencing kucing tersebut. Perkara ini mendorong penyelidikan lanjut untuk menilai implikasi zoonosis leptospirosis dalam kucing yang terdedah kepada faktor risiko persekitaran di Kelantan. Justeru, kajian ini bertujuan untuk mengetahui prevalens leptospirosis dan serovar *Leptospira* yang mengjangkiti kucing tersebut dalam kalangan kucing di Kelantan. Sebanyak 30 sampel darah (darah – 30 dan serum – 26) telah dikumpulkan daripada kucing yang dihantar ke klinik veterinar terpilih di Kelantan. Sampel serum telah diuji dengan Microscopic Agglutination Test (MAT) dan sampel darah keseluruhan tertakluk kepada Polymerase Chain Reaction (PCR). Berdasarkan keputusan, semua kucing telah diuji negatif oleh PCR. Empat kucing (15.38.%) telah diuji positif untuk MAT dengan titer antibodi ≥1:100. Daripada 04 MAT positif, 03 dijangkiti serovar Canicola dengan titer 1:100 dan 01 kucing mempunyai titer 1:200 untuk serovar Bataviae. Pengesanan antibody terhadap *Leptospira* dalam kohort kajian mempunyai implikasi yang signifikan untuk kesihatan awam dan haiwan. Keputusan serologi positif yang berkaitan dengan *Leptospira* adalah penting dalam menyumbangkan maklumat penting tentang epidemiologi penyakit tempatan, menandakan potensi peningkatan risiko leptospirosis kucing. Penemuan ini berfungsi sebagai sumber asas untuk membangunkan strategi pencegahan terhadap leptospirosis dalam kalangan kucing di Kelantan, Malaysia. Kesimpulannya, terdapat 15.38% seroprevalens leptospirosis dalam kalangan kucing yang didaftarkan untuk kajian di Kelantan.

Kata kunci: Leptospirosis, Kucing, MAT, PCR

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LIST OF ABBREVIATIONS

bp	Base pair
DNA	Deoxyribonucleic Acid
DVS	Department of Veterinary Services
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
FCV	Feline Calicivirus
FeLV	Feline Leukemia Virus
FHV-1	Feline Herpesvirus-1
FIV	Feline Immunodeficiency Virus
FPV	Feline Panleukopenia Virus
GSB	Gel Sample Buffer
HPVUMK	Veterinary Teaching Hospital of University Malaysia Kelantan
IACUC	Institutional Animal Care and Use Committee
IM	Intact Male
MAT	Microscopic Agglutination Test
OHE	Ovariohyterectomy
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
SF	Spayed female
TAE	Tris-acetate-EDTA
UMK	Universiti Malaysia Kelantan
WOAH	World Organization for Animal Health

LIST OF SYMBOLS

%	Percentage
≥	More than or equal to
µl	Microlitre
µM	Micromolar
g	Gram
ml	Millilitre
n	Number of individuals in the sample size
^{o}C	Degrees Celsius
V	Volt

CHAPTER 1

INTRODUCTION

Leptospirosis is a re-emerging zoonotic disease prevalent in tropical countries caused by the Gram-negative bacteria, *Leptospira* spp. It is a flexible, spiral-shaped, obligate aerobic spirochete with an internal flagellum that is able to infect a wide range of mammalian hosts such as humans, cats, dogs, rats, ruminants, swine, and equine species (Murillo *et al*., 2020). The genus *Leptospira* is generally classified into three groups based on pathogenicity which are pathogenic, saprophytic, and intermediate. *Leptospira interrogans* is the most common pathogenic species and consists of serovars such as Canicola, Pomona and Icterohaemorrhagiae, which are known to cause disease in humans and animals (Di Azevedo *et al*., 2021). Saprophytic species are found in soil and water and generally do not cause disease. This includes species such as *Leptospira biflexa, L. yanagawae,* and *L. meyeri* (López-Robles *et al*., 2021). Intermediate *Leptospira* spp. are of unknown pathogenicity and include *L. inadai* and *L. fainei.* (Chiriboga *et al*., 2015).

Leptospira spp. thrive in warm and humid climates of tropical countries which contributes to the endemicity of leptospirosis in such countries including Malaysia, Brazil, India, Sri Lanka, and Thailand. Humans are often infected with the pathogen after coming into contact with contaminated water and soil, especially for individuals living in areas of heavy rainfall or flooding (Ehelepola *et al*., 2019). The pathogen enters the blood circulation through broken skin or mucous membranes causing leptospiremia where it is spread to other organs causing systemic disease (Hartmann *et al*., 2013). Clinical manifestation of leptospirosis in humans differ slightly in comparison to feline leptospirosis. Human leptospirosis can present in two distinct clinical syndromes: icteric or anicteric. In icteric phase, the disease manifests as fever, severe jaundice, haemorrhage, renal failure, and respiratory distress (Wang *et al*., 2022). In contrast, cats often experience subclinical infection with minimal non-specific clinical signs which led to the belief that cats possessed an innate resistance and were unable to become infected with *Leptospira* spp. (Mazzotta *et al*., 2023). However, recent studies by Alashraf (2020) detected antibodies and successfully isolated leptospires from the kidneys of shelter cats. This not only indicates that infection is possible, but it also implies that urinary shedding is present despite lack of clinical manifestation. This prompts further research on the potential transmission of *Leptospira* spp. and zoonotic risk on cat owners.

1.1 Research problem statement

The involvement of cats in the transmission of *Leptospira* remains a topic of debate, as they are regarded both as a possible protective factor and a potential source of infection for humans. There are a few studies suggesting that cats may be acting as renal carriers and shedders of *Leptospira*. However, there is limited information about the prevalence and serovars of leptospirosis among cats in Kelantan, Malaysia. Hence, this study aims to determine the prevalence of leptospirosis and *Leptospira* serovars among cats in Kelantan.

1.2 Research Questions

1.2.1 What is the prevalence of leptospirosis among cats in Kelantan?

1.2.2 What are the most prevalent *Leptospira* species and serovars among cats in Kelantan?

1.3 Research Hypothesis

1.3.1 There is high prevalence of leptospirosis among cats in Kelantan, Malaysia.

1.3.2 The most prevalent *Leptospira* species is *L. interrogans* and serovars of *Leptospira* are Ballum, Bataviae, and Javanica that can be detected among cats in Kelantan.

1.4 Research Objectives

1.4.1 To determine the prevalence of leptospirosis among cats in Kelantan.

1.4.2 To identify most prevalent *Leptospira* species and serovars among cats in Kelantan.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of leptospirosis

Leptospirosis is one of the most prevalent (re)-emerging zoonotic disease globally and a major public health problem in many continents (Lim *et al*., 2011). The causative agent is pathogenic *Leptospira* spp., which is a Gram negative, aerobic bacteria characterised by its thin, spiral shape with hooked ends. Currently, more than 65 recognized *Leptospira* spp. that can be classified into three groups: pathogenic, saprophytic and of intermediate pathogenicity with over 250 serovars that have been identified (Yang *et al*., 2023). Classification of *Leptospira* serovars and pathogenicity is based on the cross-agglutinin adsorption test (CAAT) (Bharti *et al*., 2003). Pathogenic serovars mainly from the species *L. interrogans* are known to cause disease in humans and animals. In contrast, saprophytic species are not commonly known to cause disease and are naturally found in soil and water. These serovars are mostly from the species *Leptospira biflexa* (Mohammed *et al*., 2011). Although they are regarded as non-pathogenic, saprophytic serovar, Patoc 1 has been detected in human and feline cases of leptospirosis in Malaysia (Shafei *et al*., 2012; Alashraf *et al*., 2020).

The disease is widespread in both tropical and temperate regions, but the annual incidence is 10 times higher in tropical countries than temperate countries. Climate plays a major role in facilitating the transmission and survivability of *Leptospira* spp. in the environment. *Leptospira* spp. are destroyed through direct exposure to sunlight and desiccation at temperatures more than 34-50 ºC (Ehelepola *et al*., 2019; Mohammed *et al*., 2011), hence it thrives in warm and humid weather. This contributes to its endemicity in certain tropical countries such as Malaysia, Sri Lanka, Thailand, Brazil, and India (Soo *et al*., 2020). These countries often experience periods of heavy rainfall during monsoon season which also contributes to flooding and a sudden increase in leptospirosis cases in humans and animals. This is caused by the heavy rainfall which allows contaminated urine to flow into bodies of stagnant water and moist soil where they can stay viable for several months (Mohammed *et al*., 2019). Yaakob *et al*., (2015) reported a total of 110 cases of leptospirosis in humans after a major flood in Kelantan, Perak, and Terengganu, in 2014 due to the leaching of *Leptospira* spp. into the soil.

2.2 Leptospirosis in animals in Malaysia

Almost every mammalian species is susceptible to *Leptospira spp*. infection and can effectively shed the pathogen into the environment through urine. In Malaysia, 38 leptospiral serovars that had been isolated and identified from animals (Garba *et al*., 2017; Ridzlan *et al*., 2010). Several studies in Malaysia have investigated leptospirosis in various animal species, including dogs, cats, ruminants, and swine.

Cattle are considered major reservoirs for *Leptospira* spp. and is a major contributor of reproductive disorders in cattle including early embryonic death, stillbirths, and abortion (Adugna, 2016). Bovine leptospirosis among cattle is regarded as a notifiable disease in Malaysia (DVS, 2011). In a study by Sabri *et al*., (2020), 1024 ruminants in Kelantan exhibited a prevalence of 11.75% and the common serovars being Hardjo-bovis, for cattle and goats, and Hebdomadis for sheep. These serovars were detected from blood samples from ruminants reared in flood-prone areas. Similar to other species, leptospires are capable of producing pathological changes to the kidneys as reported by Kamaruzaman *et al*., (2023) which reported severe diffuse necrotizing glomerulonephritis and tubulointerstitial nephritis in bovine kidneys sampled from local wet markets in Kelantan. In the same study, *L. interrogans* and *L. borgspetersenii* were able to be detected from the kidney samples. This indicates that cattle are also able to shed pathogenic *Leptospira* spp. through the urine and impose a zoonotic risk to cattle farmers in Malaysia (Kamaruzaman *et al*., 2023).

There is a paucity in research on leptospirosis in swine in Malaysia despite its ability to cause major economic losses in the swine industry and the occupational risk it imposes on local farmers. Chronic leptospirosis can produce similar clinical signs in ruminants whereby there will be reproductive failure, abortion, stillbirths, and smaller litter sizes (Zhitnitskiy, 2015). In 2017, it was reported that the predominant serovars detected in three swine farms in Selangor were Pomona and Bratislava, with a prevalence of 6% (Benacer *et al*., 2017).

Studies conducted in 2015 and 2016 among dogs in Klang Valley identified Canicola, Bataviae, and Icterohaemorrhagiae as the common serovars infecting both healthy and shelter dogs (Lau *et al*., 2016; Hua *et al*., 2016). Another study among working dogs throughout Malaysia reported a prevalence of 3.1%, with Australis, Bataviae, and Javanica as the common serovars (Lau *et al*., 2017). It is important to note that the core vaccination for dogs includes a leptospiral component against Pomona, Icterohaemorrhagiae, Canicola, and Grippotyphosa (Lau *et al*., 2017). Among the various

groups of dogs in Malaysia, shelter dogs exhibit the highest prevalence of leptospirosis. Further study of this group is warranted, as they are likely to best represent the *Leptospira* spp. present in the environment due to their lack of vaccination.

2.3 Leptospirosis in cats

The initial documentation of feline leptospirosis dates back to 1972. In a study by Mazzotta *et al*., (2023) it was reported that cats were resistant to both natural and experimental infections. However, there have been more recent studies that contradict this. In 2020, there was the first report of pathogenic *Leptospira* spp. isolated from the urine and the kidneys of naturally infected shelter cats in Malaysia. In the study, 21/82 (25.61%) cats were positive for leptospirosis using MAT while 4/82 (4.9%) of urine samples and 7/82 (8.5%) whole blood samples contained *Leptospira* spp. DNA as detected by PCR (Alashraf *et al*., 2020). This indicates leptospiuria and leptospiremia is possible in cats despite lack of clinical signs and indicates the role of cats as subserological and subclinical reservoirs of the bacteria.

Prevalence studies in Spain indicate that the primary serovars associated with leptospirosis in cats is belonging to the serogroups Australis, Autumnalis, Canicola, and Sejroe (Murillo *et al*., 2020). However, research studies on leptospirosis among shelter cats in Malaysia are scarce and there are a few studies conducted in Selangor and Johore which identified the common serovars as Ballum, Bataviae, and Javanica with the prevalence of 18.18% and 25.6% (n=20/110) (Alashraf *et al*., 2019). In the same study, *L. biflexa* serovar *Patoc1* were detected from blood samples in 7/82 cats indicating that there can be co-infection with more than one serovar and species.

Leptospiral infection in cats has been linked to the ingestion of prey (rats) that is infected with the leptospires. Outdoor cats face a higher risk of contracting leptospires due to their proximity to reservoir hosts. In rural regions, cats can also acquire the infection through contact with urine from pigs and cows. Notably, having another cat in the household significantly elevates the risk of seropositivity for leptospirosis. A review by Murillo *et al*., reported that the prevalence of *Leptospira* DNA shedding in urine among cats is ranged from 0% to 67.8%, with no clear association with clinical disease.

2.4 Pathogenesis of leptospirosis

The pathogenesis of feline leptospirosis is similar to infection in dogs and humans where the pathogen gains entry after the host comes in either direct contact with infected

urine or indirect contact with contaminated soil or water. The pathogen enters through mucous membranes or through injured skin and enters the bloodstream to cause leptospiraemia, where the bacteria circulate in the blood (Adler *et al*., 2014). Within one day, the pathogen multiplies rapidly within the bloodstream and spreads to organs such as the liver, spleen, and kidneys. In the organs, the presence of the bacteria triggers inflammation and injury to the tissue, especially the kidneys and liver (De Brito *et al*., 2018). The pathogen can persist within the kidneys and the host can continuously shed the bacteria through the urine despite the resolution of the infection. Clinical signs of leptospirosis in cats may appear in some instances which include fever, lethargy, loss of appetite, vomiting and in severe cases, jaundice. Kidney involvement can lead to renal failure.

2.5 Diagnosis of leptospirosis

Blood and urine are the biological samples that can be used in laboratory diagnosis of leptospirosis. Laboratory diagnosis of leptospirosis could be accomplished by either direct or indirect assays. Direct laboratory techniques involve the detection of *Leptospira* through microscopic observation, culture isolation and detection of *Leptospira* DNA by molecular methods such as Polymerase Chain Reaction (PCR). PCR targets either housekeeping genes which are present in all pathogenic, saprophytic, and intermediate *Leptospira* species or specific genes that are only encoded in pathogenic species. This method is rapid and accurate in detecting the bacteria; however, it cannot be used as a lone diagnostic method as leptospiremia is often transient and negative results does not indicate absence of infection. Bacterial isolation on selective growth medium, Ellinghausen McCullough Johnson Harris (EMJH), of blood and/or urine is not commonly employed for diagnosis due to its time-consuming nature and susceptibility to contamination by ubiquitous saprophytic leptospires (Ridzlan *et al*., 2010).

Microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) are two important indirect diagnostic assays that are widely used in diagnosis of leptospirosis which use serum samples to detect the presence of pathogen-specific antibodies. MAT is considered as the gold standard for diagnosis of leptospirosis (Goris, M. G., 2014). MAT is useful in diagnosis of the disease as well as identifies the specific serovar infecting the individual, providing better approach for epidemiological evidence. However, it should be noted that there has not been a commercial rapid test kit or ELISA kit developed for the diagnosis of leptospirosis in cats. This is reflective of the belief that

cats are resistant to the disease leading to the underreporting of the disease in cats and low demand for such a diagnostic kit (Andityas *et al*, 2022).

2.6 Zoonotic Impact of leptospirosis

In Kelantan, cats are the most commonly kept pets that live in closest proximity to humans compared to dogs primarily due to cultural and religious barriers. In Kelantan, they are commonly managed as semi-roamers by which the animal spends an equal amount of time indoors and outdoors. Semi-roamers and fully outdoor cats have an increased risk of being exposed to *Leptospira* due to close contact with reservoir hosts harbouring the pathogen such as rats, pigs, and cattle (Murillo *et al*., 2020; Dorsch *et al*., 2020).

Most infections in cats are subclinical and infected cats do not show prominent clinical signs. Reports of feline leptospirosis describe an acute onset of polyuria, polydipsia, anorexia, lethargy and in some cases, haematuria (Arbour *et al*., 2009). Blood analysis also did not reveal any significant parameters other than marked leucocytosis and increased liver enzymes. Hence, owners might handle urine without proper precautions since their cat appears somewhat healthy, posing a zoonotic risk to their owners.

A study conducted in Selangor and Johore determined a prevalence of 18.18% among shelter cats and identified Bataviae and Javanica as the common serovars infecting the cats as reported by A.R Alashraf *et al*., 2018. It is noteworthy that the serovars most involved in 84 reported human leptospirosis cases from a Malaysian hospital are Bataviae and Javanica (Rafizah, *et al*., 2013) and the same serovars were also found to infect the rat population in Kuala Lumpur (Alashraf *et al*., 2019). Various studies also depict possibility of leptospiuria in cats despite lack of clinical signs and this urinary shedding can be a source of infection for cat owners considering the fact that cats can shed the pathogen through urine for up to 8 months (Weis *et al*., 2017).

CHAPTER 3

METHODOLOGY

3.1 Ethics approval

The ethical clearance for the study was obtained from the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (FPV, UMK) with the approval code of UMK/FPV/ACUE/FYP/006/2023.

3.2 Acquiring consent and subject medical history

Owners and subjects were approached at the Veterinary Teaching Hospital, Universiti Malaysia Kelantan (HPVUMK) and selected private veterinary clinics within Kota Bharu. Prior to blood sample collection, a brief explanation of the study, the procedure and the possible complications for sample collection was explained to the owners. Once the owners agreed, they sign the consent form and contact information was collected (Appendix A). Information regarding the patient's signalment, management and medical history was obtained using the study tool (Appendix B). The collected data were tabulated for further analysis.

3.3 Sample collection

A total of 30 blood samples were collected from cats during the period from September to October 2023. Approximately 2 ml of blood were obtained by a licensed veterinarian following universal precautions. The blood specimen (1 ml) was collected into a plain blood collection tube for serum separation and additionally 1 ml blood was collected into an Ethylenediaminetetraacetic Acid (EDTA) blood collection tube for DNA extraction procedures. All tubes were appropriately labelled and promptly placed in an ice box for transportation to the laboratory.

Blood samples collected in plain blood collection tube were allowed to clot by keeping for 30 minutes at room temperature and then centrifuged at 3,800 rpm for 15 minutes for serum separation. The serum was collected using a sterile pasteur pipette and aliquoted into sterile microcentrifuge tube and stored at -20 ^{o}C. EDTA blood tubes were stored at -20 ^{o}C for DNA extraction procedures.

3.4 Molecular detection of *Leptospira* spp.

3.4.1 DNA Extraction

DNA extraction was performed using a commercial DNA extraction kit (Geneaid gSYNC™) following the manufacturer's instructions. EDTA tubes were taken out of storage and allowed to thaw at room temperature for 30 minutes prior to extraction. A 200 µl aliquot of whole blood was transferred into a 1.5 ml microcentrifuge tube. In cases where the blood volume was insufficient, phosphate buffered solution (PBS) was added until a total volume of 200 µl was achieved. Subsequently, 20 µl of proteinase K was added and mixed with the sample in the microcentrifuge tube which was then incubated in a water bath at 60°C for 10 minutes.

Following the initial incubation, 200 µl of GSB buffer was added to the tube, which was vortexed and incubated at 60°C for 10 minutes. To this mixture, 200 µl of absolute ethanol was added and immediately vortexed for 10 seconds. A GS column was positioned in a 2 ml collection tube, and the entire mixture from the microcentrifuge tube was transferred. The 2 ml collection tube was centrifuged at 14,000 rpm for 1 minute until all the mixture passed through the GS column. 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, 400 µl of W1 Buffer was added to the GS column and centrifuged for a second time at 14,000 rpm for 30 seconds. The resulting flow-through was discarded. The GS column was reinserted into the 2 ml collection tube and 600 µl of wash buffer was added. It was then centrifuged at 14,000 rpm for 3 minutes to ensure the column was completely dry. Finally, the dried GS column was transferred to a microcentrifuge tube and 100 µl of pre-heated elution buffer was added. The tube was then centrifuged at 14,000 rpm for 3 minutes to elute purified DNA. The microcentrifuge tube with extracted DNA was labelled and stored in a cryobox at -20 °C.

3.4.2 Polymerase Chain Reaction (PCR)

The molecular detection of *Leptospira spp.* was performed by conventional PCR using two sets of primers which target two different regions of the *Leptospira spp.* genome. Table 3.4.2.1 shows the list of primers used in this study.

Table 3.4.2.1: List of primers used in this study

Primer	Targeted genes	Product size (bp)	Sequences (5' – 3')	Sources
16s rRNA	*Leptospira* spp.	330	F: 5'- GGC GGC GCG TCT TAA ACA TG – 3' R: 5'- TCC CCC CAT TGA GCA AGA TT-3'	Merien *et al.* (1992)
LipL32	Pathogenic gene	~700	F: 5'- TTA CCG CTC GAG GTG CTT TCG GTG GTC TGC-3' R: 5'- TGT TAA CCC GGG TTA CTT AGT CGC GTC AGA -3'	Chaemchuen *et al.* (2011)

Merien et al. (1992); Chamchuen et al. (2011).

A 25 µl PCR reaction mixture containing 12.5 µl 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. In this study, 5 µl DNA sample was then added to the tube. The condition of each PCR reaction was carried out using the published studies with slight modifications. Positive and negative controls were included for each experiment.

Temperature profile as one cycle for 16S rRNA fragment were 95˚C for 5 minutes, 34 cycles at 95˚C for 30 seconds, 60.4˚C for 30 seconds, 72˚C for 1 minute and final elongation at 72˚C for 5 minutes. PCR targeting LipL32 gene was performed only if PCR for 16s rRNA was positive.

LipL32 gene were amplified by the initial one cycle of 95˚C for 5 minutes, 34 cycles at 95˚C for 30 seconds, 60˚C for 30 seconds, 72˚C for 1 minutes and final extension at 72˚C for 5 minutes, respectively.

3.4.3 Gel Electrophoresis

The amplified products were evaluated by agarose gel electrophoresis. A 2% (w/v) agarose gel was prepared first by mixing 1.2 g of agarose powder in 60 ml of TBE buffer in a schott bottle and microwaved for 2 minutes until the agarose was completely dissolved. Agarose solution was allowed to cool down and 0.1 µl of Midori Green dye was added into the solution. The agarose solution was then poured into the gel tray with well comb in place. The gel was placed at room temperature for 20 minutes until it had completely solidified.

Once solidified, the agarose gel was placed into the electrophoresis tank, which was covered with 10% (v/v) TBE buffer. A 5 µl 100 bp DNA ladder was loaded into the first lane of the gel. The PCR products were then loaded to the additional wells of the gel, which the last two wells were loaded with positive control (*L. interrogans*) and negative control (distilled water). The electrophoresis gel was then run at 100 V for 40 minutes. The DNA fragment was then visualized using GelDocTM EZ Imager which the DNA fragment was appeared as band on the gel. By using molecular weight 100 bp DNA ladder as the guide, the size of the DNA products was determined.

3.5 Microscopic Agglutination Test (MAT)

Serum samples were sent to Bacteriology Laboratory, Veterinary Laboratory Services Unit, Department of Veterinary Laboratory Diagnostics, Faculty of Veterinary Medicine, Universiti Putra Malaysia to obtain single MAT antibody titres. MAT titres were obtained using a panel of six Leptospira serogroups namely Canicola, Bataviae, Javanica, Australis, Ballum and Autumnalis. MAT titre of ≥1:100 was used as the cut off value to be considered as positive as recommended by the Department of Veterinary Services (DVS) for the diagnosis of leptospirosis in animals in Malaysia (DVS Malaysia, 2011).

3.6 Statistical Analysis

Data collected during the study using the study tool in Appendix B were recorded and tabulated into a database and subsequently analysed using Microsoft Excel. The ratio of incidence of the risk factors that may contribute to the prevalence of leptospirosis were calculated.

CHAPTER 4

RESULTS

4.1 Demographic data

Demographic data for enrolled cats (n=30) were tabulated in Table 4.1.1, encompassing data such as sex, age, management, and place of residence.

The age of the study participants ranges from 7 months to 4 years old, with the mean of 12 months (*SD=11.57).* The majority of the cats in the study are reared as semi-roamers (54.54%). The second most common group comprises cats reared fully outdoors (36.36%), while the least common group consists of cats reared fully indoors, with only 3 (9.09%) participants falling into this category. Moreover, the majority of the participants were found to be intact, with only 6.06% having been castrated or spayed. In terms of residence, 78.79% of the cats live in urban areas, while 21.21% reside in rural areas. Ten (33.33%) cats enrolled in this study were found to be experiencing disease conditions ranging from mild diseases such as conjunctivitis, fungal infection, and mange to more severe disease conditions such as parvoviral infection, persistent haematuria, liver damage and hindlimb fracture requiring amputation. The severity of the clinical signs also varied between the cats whereby some showed mild clinical signs such as sneezing and purulent nasal discharge with sneezing. These clinical signs were present in two cats with conjunctivitis and mange infestation. One cat had past history of liver damage but has since received treatment and the condition has mostly resolved hence, the cat was mostly healthy despite its condition. The same findings were noted for the cat with hindlimb fracture whereby other than the limiting hindlimb fracture, he showed no other significant clinical signs. A majority (66.66%) of the cats were healthy and mostly presented to the clinic for routine medical procedures or for routine ovariohysterectomy (OHE) or castration.

Table 4.1.1 : Demographic data of the participants

Sample characteristics	*n*	*%*
Sex		
Castrated male	2	6.06
Spayed female	2	6.06
Intact male	13	39.39
Intact female	16	48.48
Age (months)		
~7-12 months	12	36.36
~13- 24 months	11	33.33
~25-48 months	10	30.30
Management		
Indoors	3	9.09
Outdoors	12	36.36
Semi-roamer	18	54.54
Place of residence		
Urban	26	78.79
Rural	7	21.21
Health status		
Healthy	20	66.66%
Diseased	10	33.33%

4.2 Polymerase Chain Reaction (PCR)

Amplification of the 16S rRNA of *Leptospira* spp. showed that all the samples were negative for PCR. Figure 4.1.1 and Figure 4.1.2 shows PCR result for sample 1-30.

PCR for LipL32 primer was not performed in this study as all the samples were negative for 16S rRNA primer**.**

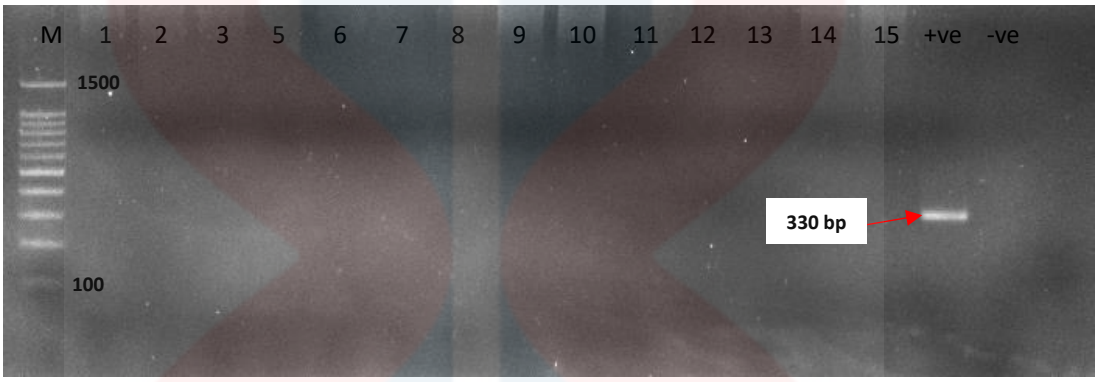

Figure 4.2.1: PCR amplification of 16S rRNA gene of Leptospira spp. demonstrated by agarose gel electrophoresis. M: 100bp ladder; lane 1 to 15: Samples; +ve: positive control; -ve: negative control.

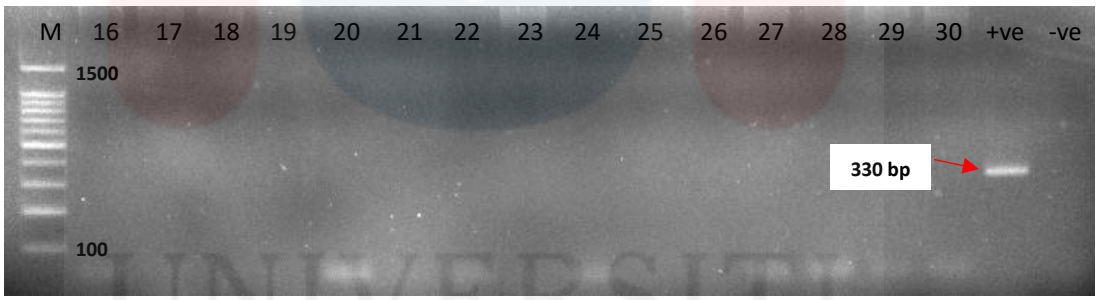

Figure 4.2.2: PCR amplification of 16S rRNA gene of Leptospira spp. demonstrated by agarose gel electrophoresis. M: 100bp ladder; lane 16 to 30: Samples; +ve: positive control; -ve: negative control.

4.3 Microscopic Agglutination Test (MAT)

From the total of 26 serum samples tested using MAT, a total of 04 samples were tested positive based on the cut-off point of ≥1:100, indicating a seroprevalence of 15.38%. The results revealed that 03 cats were positive for serovar Canicola with the titre of 1:100 while only one sample was tested positive for serovar Bataviae with the titre of 1:200. Hence, the predominant serovar in this study is serovar Canicola. The resulted antibody titre of MAT according to the MAT panel used in the study are tabulated in Table 4.3.1 below while Table 4.3.2 shows the background information regarding the cats testing positive for MAT.

Table 4.3.1: Summary of MAT results

Serovar	Titre	Number of cats positive
Canicola	1:100	3
Bataviae	1:200	1
Australis	-	0
Javanica	-	0
Ballum	-	0
Autumnalis	-	0

Table 4.3.2*:* Background information of the cats testing positive for MAT

Subject	Serovar	MAT Titre	Age (years)	Sex	Management	Origin	Medical condition	Clinical signs
Cat 1	Canicola	1:100	3	IM	SR	Rural	Fungal and mange	Purulent nasal discharge
Cat 2	Canicola	1:100	4	SF	I	Urban	CKD	Haematuria
Cat 3	Canicola	1:100	1	IM	SR	Urban	-	-
Cat 4	Bataviae	1:200	1	IM	O	Urban	-	-

IM: Intact male, SF: Spayed female, SR: Semi roaming, I: Indoor, O: Outdoor, CKD: Chronic kidney disease

CHAPTER 5

DISCUSSION

Leptospirosis is a re-emerging disease in Malaysia affecting humans and domestic animals, especially in areas prone to flooding. This renders the disease an important topic of discussion in certain states such as Kelantan, Terengganu, Perak, and Pahang (Yaakob *et al*., 2015). Due to certain cultural and religious beliefs of the residents of Kelantan, particular emphasis on the possible transmission of leptospirosis between cats and humans should be researched further as they commonly live in close proximity to their owners. This is reflected in the study whereby semi-roamers made up the majority (54.54%) followed by outdoor cats (36.36%) and indoor cats (9.09%). Semi-roamers pose a great threat to their owners as they often have a greater likelihood of being exposed to environmental risk factors (Rodriguez *et al*., 2014). In semi-roamers and fully indoor cats, owners often handle the urine and faeces of their cats which might be a possible route of transmission of leptospirosis in humans if proper precautions are not taken. However, this has not been demonstrated in any study thus far. This would be an area of research in the future to definitively determine the possibility of cats transmitting leptospirosis to their owners.

Current study enrolled 30 cats by collecting blood samples (whole blood – 30 and serum – 26) which were subjected to PCR (n=30) and MAT (n=26). For MAT, 04 of 26 samples were positive while PCR revealed a negative result for all 30 samples. As stated by WOAH (2021), the gold standard for the diagnosis of leptospirosis is MAT which requires serum sample for the detection of specific antibody towards leptospires. Serum as a sample is more accurate as antibodies are found as early as 12 days after infection (Shropshire *et al*., 2016) and lasts for approximately several weeks to months (WOAH, 2021). In the present study, it was detected that 4 out of the 26 cats tested for MAT were confirmed to be positive for leptospirosis with the predominant serovar being Canicola followed by serovar Bataviae with the prevalence being 15.38%. Out of the four cats tested positive for MAT, two cats were stray cats that were brought in for neutering and were mostly healthy. However, one of these cats tested positive for Canicola with MAT titre of 1:100 while the other was the only cat tested positive for Bataviae with MAT titre of 1:200. Both of these cats were intact males and were collected from areas with known rat infestations and areas prone to flooding hence, it is highly likely that these cats

contracted leptospirosis from the environment. This is supported by the findings of a study carried out to detect the prevalence of leptospirosis in rats infesting wet markets in Kelantan which produced a prevalence of 72% using PCR with kidney samples. Sequencing from these positive results revealed that a majority of the positive samples were found to be infected with pathogenic *L. interrogans* (Kamaruzaman *et al*., 2022). However, specific serovar was not determined as MAT was not performed. Another study detected two of 81 rats were tested with MAT and produced a positive result for Canicola in a National Service Training Centre in Kelantan (Mohamed-Hassan *et al*., 2010). However, in this study the cut-off point of ≥1:40 was used instead of ≥1:100 and the titre for Canicola was not disclosed. Hence, it is possible that the seropositive cats in the current study acquired the infection from hunting rats in the surrounding area.

As stated by Mazzotta *et al*., 2023, presence of immunosuppressive co-morbidities including feline herpesvirus (FHV-1), feline leukemia virus (FeLV), feline panleukopenia virus (FPV) and feline immunodeficiency virus (FIV) greatly increased the possibility of contracting leptospirosis especially in young cats. This was reflected in the present study whereby one seropositive 3-year-old intact male residing in a rural area with notable rat infestation. Although the cat was not definitively diagnosed with the aforementioned diseases, it can be presumed that the cat possessed one or more of the immunosuppressive viruses as the cat did not receive its core vaccinations against feline calicivirus (FCV), FHV-1, and FPV. The cat also had severe fungal skin infection and mange which may be indicative of the presence of an immunosuppressive disease (Frymus *et al*., 2013).

Notably, of the four seropositive cats, one cat presented to the hospital for its chronic kidney disease and was noted to be experiencing persistent haematuria for several months. This particular cat had a MAT titre of 1:100 for the serovar Canicola. It should be noted that this cat was reared completely indoors in a multi-cat household with six other cats in an urban area and does not venture out of the house due to its condition. Owner also noted that the cat does not often come into contact with rats or drink water outside. It is possible that the infection was obtained from the other cats within the household that might have been recently adopted. Rodriguez *et al*., 2014, noted that there was increased risk of seropositivity in a multi-cat household and the hypothesized route of transmission between these cats is through litter box sharing (Rodriguez *et al*., 2014). In the same study, it was noted that natural infection of *Leptospira* spp. in cats were more commonly detected in cats with chronic kidney disease (14.9%) than in healthy-

presenting cats (7.2%). This is supported by Shropshire *et al*., 2016, which stated *Leptospira* spp. were also noted to be detected more in cases with clinical signs relating to the renal system such as polyuria, polydipsia, and haematuria. During its presentation to the hospital, the cat did not show any clinical signs relating to leptospirosis other than haematuria. Hence, it is unknown whether the cat is experiencing current clinical leptospirosis or possesses antibodies against *Leptospira* spp. due to a past infection. To verify the cat's carrier status and the zoonotic risk imposed on its owner, further analysis with PCR using urine sample is recommended in the future. In the meantime, the owner would be advised to handle the urine with appropriate precautions such as by wearing gloves and ensuring proper disposal as this was noted to be a possible method of transmission between cats and their owners (Sprißler *et al*., 2019).

Despite most of the participants within the study fulfilling most of the risk factors for leptospirosis, the PCR results were negative for all 30 samples. This indicates the cats were not in the leptospiraemic phase at the time of sample collection. However, leptospiraemia is often transient and according to Murillo *et al*., (2020) leptospiraemia occurs in the acute phase of infection and lasts for only 3-7 days in the blood. The study also suggests urine as the more suited sample for diagnosis of leptospirosis especially in cats due to their lack of clinical manifestation even in the acute phase of infection. This sample was not obtained in the present study as urine sample collection is often done by manual compression. However, cats often do not tolerate this method of collection and resist compression of their bladder. This causes an insufficient volume of urine that is able to be collected. To overcome this issue, manual compression should be performed under general anaesthesia. This was impractical to perform for this present study due to time constriction. However, in future studies, urine should definitely be considered as an additional and better suited sample for molecular detection of *Leptospira* spp. This is further supported by the findings of a study in Selangor conducted by Alashraf *et al*., which detected presence of *Leptospira* spp. DNA through PCR within kidneys and urine from shelter cats in Malaysia. This is also the preferred method of diagnosis as leptospires persist within urine for up to 8 months (Weis *et al*., 2017). Hence, urine would be recommended as a future sample.

CHAPTER 6

CONCLUSION

This study is the first report of feline seropositivity locally in Kelantan. Despite small sample size, a high seroprevalence was detected among cats in Kelantan whereby 15.38% were tested positive for leptospirosis by detecting Canicola and Bataviae as two serovars infecting cats in the study. Nevertheless, it is imperative to conduct planned and region-specific studies to gain valuable insights into the epidemiology of leptospirosis in the feline population of Kelantan. Our findings offer proof that seemingly cats might function as subclinical and sub-serological reservoirs of leptospires. This awareness could potentially stimulate the formulation of effective disease prevention strategies for the feline population. Further investigations, including extensive molecular analysis incorporating larger sample size and different samples such as urine are warranted in future studies.

CHAPTER 7

RECOMMENDATIONS

This study was limited by a small sample size leading to difficulty in analysis of data which may distort the findings of the data analysis which determines relationship between different variables within the study. Hence, it would be advisable to increase the sample size of the study to allow for better representation of the cat population of Kelantan and produce more accurate statistical results. However, despite this limitation, the data is able to provide baseline data for a more extensive study in the future.

During the sample collection, a large majority of the cats were uncooperative and struggled during blood collection. This formed imperfect serum whereby it possessed a red discoloration instead of the expected straw-coloured serum. This may affect the results obtained from the diagnostic tests. Hence, it would be recommended for blood samples to be collected prior to a medical or surgical procedure while the cat is under general anaesthesia. This would ensure both the quality and quantity of the blood samples obtained. Urine sample collection can also be performed in these patients as urine sample are the best suited sample to indicate the cat's carrier status.

Lastly, in future studies, it would be recommended to incorporate both whole blood samples and urine samples or kidney samples for PCR. Positive results for urine samples would indicate the cat's carrier status as well as indicate presence of urinary shedding. This would allow for the owners to take the appropriate precautions during the handling of their pets' urine. In addition, positive samples detected from urinary shedders should be confirmed by collecting serum samples from their owners for testing with MAT to further confirm possible transmission between cats and their owners.

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APPENDIX A

UNIVERSITI MALAYSIA KELANTAN

FACULTY OF VETERINARY MEDICINE UNIVERSITI MALAYSIA KELANTAN

Borang Persetujuan Pelanggan

Tajuk Penyelidikan: Pengesanan Leptospirosis dalam kalangan kucing di Kelantan.

Kami ingin menjemput anda untuk mengambil bahagian dalam kajian untuk mengesan kehadiran Leptospira pada kucing.

Untuk menjalankan kajian ini, kami ingin mendapatkan persetujuan anda untuk mengumpul sampel darah daripada kucing untuk digunakan bagi ujian serologi dan ujian molekul. Pengumpulan sampel akan dikendalikan oleh doktor haiwan yang berdaftar.

Setiap langkah akan diambil untuk memastikan proses pengumpulan sampel dilakukan dengan cermat dengan risiko yang minimum atau sifa kepada kucing peliharaan.

Jika anda memutuskan untuk mengambil bahagian dalam projek ini, kami menawarkan ujian diagnostik Leptospirosis percuma anggaran RM200 untuk kucing anda.

Kami akan memastikan bahawa semua maklumat peribadi yang diperoleh akan dirahsiakan. Kami akan memaklumkan kepada anda mengenai keputusan ujian diagnostik yang dijalankan kerana Leptospirosis merupakan penyakit zoonotik.

Terima kasih atas penyertaan anda dan telah membantu kami dalam kajian ini.

Saya secara sukarela bersetuju untuk mengambil bahagian dalam projek ini. Saya faham bahawa saya boleh menarik balik persetujuan saya pada bila-bila masa. Saya dengan ini memberi persetujuan untuk penyertaan dalam projek ini.

Nama :_____Nama Haiwan:_____No.

Case: _____ Tandatangan: _____

Email : _____ Tarikh: ___

Individu untuk dihubungi sekiranya timbul sebarang masalah: Dr. Thilini Nisansala (019-8909753) / Dr. Mohammad Sabri Bin Abdul Rahman (013-6339874) / Wan Jazmina Binti Wan Aasim (012-9086865)/ Email address: thilini@umk.edu.my / sabri.ar@umk.edu.my / jazmina.d19a0042@siswa.umk.edu.my

APPENDIX B

Detection of Leptospirosis among Cats in Kelantan

Case number: ……..... Date: ……

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

Pet's Information

1. Cats's Name: ……
2. Cat's Age: ……
3. Cat's Breed: ……
4. Sex of the Cat: Male ☐ Female ☐ Entire ☐ Sterilized ☐
5. Place of residence: Urban area ☐ Rural area ☐
6. Housing of your Cat: 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.

   Cats ……

   Dogs ……....

   Other (Specify) ……
8. Cat's vaccination status: Yes ☐ No ☐

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

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

   Date of last vaccination against leptospirosis: ……..
9. Does your cat has any medical condition, Yes ☐ No ☐

   If yes, what is the condition? ……
10. Is your cat receiving any medication currently? Yes ☐ No ☐

    If yes, which medications? ……
11. Has your cat received antibiotics during the past six months? Yes ☐ No ☐
12. Does your cat come into any contact with rats? Yes ☐ No ☐
13. Does your cat drink water outside? Yes ☐ No ☐

    If yes, where? …….. (puddles, ditches, lakes, sea etc.)
14. Does your cat have any recent outdoor/recreational activity? Yes ☐ No ☐

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

15. Has your cat expose to flooding recently?

If yes, month and year of exposure ……

Samples obtained.

Blood ☐