MOLECULAR DETECTION OF *LEPTOSPIRA* SPP. IN BOVINE KIDNEY IN KOTA BHARU, KELANTAN

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Molecular detection of *Leptospira* spp. in bovine kidneys in Kota Bharu Kelantan'** by Ong Siew Zee, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 5436 – Research Project.

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Thank You

DEDICATIONS

I would be honour to dedicate my dissertation work to my family and friends. A special feeling of gratitude to them, whose words of encouragement and push the tenacity ring in my ears.

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Abbreviations

BL	Bovine leptospirosis
ELISA	Enzyme linked immunosorbent assay
H&E	Haematoxylin and eosin
MAT	Microscopic agglutination test
PCR	Polymerase chain reaction

ABSTRACT

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

Leptospirosis is a bacterial disease that has zoonotic potential, affecting both humans and animals. The aim of this study was to detect the *Leptospira* spp. and determine the predominant species related in the bovine kidney samples collected from selected wet markets in Kota Bharu, Kelantan. Furthermore, to investigate the pathological alterations of bovine kidneys related to the disease. A total of 50 bovine kidney samples were collected from four wet markets across Kota Bharu area and the samples were tested using polymerase chain reaction (PCR) assay. From the results, five samples (10%) were found to be positive for 16S rRNA gene, while one (2%) sample showed positive results for LipL32 gene. The further sequencing of the positive samples for both 16S rRNA and LipL32 gene were revealed as *Leptospira borgpetersenii*, which is known to be a pathogenic species in cattle. Furthermore, the bovine kidneys that gave positive PCR results showed relevant histopathological lesions of bovine leptospirosis, consisting of interstitial nephritis, glomerular atrophy and tubular necrosis. In conclusion, this study demonstrated the presence of pathogenic *Leptospira* spp. in bovine kidneys, which caused a series of histopathological changes and could pose zoonotic potential to the public.

Keywords: *Cattle, Histopathology, Leptospirosis, LipL32, PCR, 16S rRNA*

ABSTRAK

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

Leptospirosis adalah merupakan satu penyakit zoonosis yang disebabkan oleh bakteria *Leptospira* spp. yang menjejaskan manusia dan haiwan. Kajian ini adalah bertujuan untuk menentukan spesis *Leptospira* spp. dalam ginjal lembu daripada pasar-pasar terpilih sekitar Kota Bharu, Kelantan serta mengkaji perubahan histopatologi yang berkaitan dengan penyakit ini. Oleh itu, sejumlah 50 ginjal lembu dikumpulkan dari empat pasar di seluruh kawasan Kota Bharu, Kelantan, dan diuji menggunakan tindak balas berantai polimeras (PCR). Hasilnya, lima (10%) ginjal didapati positif untuk gen 16S rRNA dan 1 (2%) ginjal ditentukan positif dengan gen LipL32. Keputusan penjujukan DNA menunjukkan bahawa sampel-sampel tersebut adalah identikal kepada *Leptospira borgpetersenii* yang merupakan species patogenik pada lembu. Selain itu, perubahan histopatologi, seperti interstitial nefritis, nekrosis tubul dan atrofi glomerular dapat dikaji dalam ginjal yang positif leptospirosis. Kesimpulanya, kajian ini dapat menentukan spesis *Leptospira* dalam ginjal lembu yang menyebabkan perubahan histopatologi dan mempunyai potensi zoonosis kepada orang awam.

Kata kunci: *Histopatologi, Lembu, Leptospirosis, LipL32, PCR, 16S rRNA*

1.0 Introduction

Leptospirosis is one of the most prevalent zoonotic disease found worldwide (Levett, 2004) which is caused by pathogenic bacteria species within the genus *Leptospira*. In Malaysia, leptospirosis is an endemic disease due to its tropical climate. *Leptospira* spp. can survive in aquatic environments such as ponds, river, surface waters, moist soil and mud when temperatures are moderate. Hence, the warm and humid weather in Malaysia allow this organism to thrive longer in the environment and increase the risk of exposure. This contributes to the dramatic increase of the number of reported cases in Malaysia. According to the Ministry of Health Malaysia, a marked increase of leptospirosis occurred from 12.5 per 100,000 population in 2012 to 15.0 per 100,000 population in 2013 (Benacer et al., 2016).

Leptospirosis has a major public health concern in view of its occurrence in human and in animals, including the wild and domestic animals. Many species of domestic animals serve as carriers or hosts for *Leptospira* spp., including the livestock animals such as cattle, goat and sheep. Among these livestock, cattle has one of the highest prevalence (Abdul Rahman et al., 2021). Currently, 65 recognized *Leptospira* spp. are classified into three groups, which are pathogenic, non-pathogenic and intermediate pathogenicity with more than 250 serovars identified (Cerqueira and Picardeau, 2009; Monroy et al., 2021). In Malaysia, there are 38 leptospiral serovars that had been isolated and identified from animals. The most frequent leptospiral serovars detected in livestock in Kelantan, Malaysia were Hardjo, Hebdomadis and Pomona (Bahaman 1988; Abdul Rahman et al., 2021).

The pathogenicity of *Leptospira* spp. related to the virulence of the infecting serovar and the susceptibility of the host species (Quinn et al., 2011). Following the systemic infection, certain leptospira organisms are cleared out from the circulation by the antibodies at about 10 days after infection. However, the bacteria may evade the immune response, colonizing the renal tubules, which is an antibody free site. Renal colonization leads to the damage of the kidney and occurrence of leptospiruria. Animals with colonized of *Leptospira* spp. in their renal tissues can shed live organisms in urine, contaminating the environment. Live leptospira organisms may transmitted to the susceptible hosts, including human via direct contact of bodily fluid of the infected animals or indirectly via contaminated water or soil source.

In the recent years, studies on bovine leptospirosis (BL) in Malaysia is very limited especially in the north-eastern region. Hence, this study was carried out to detect the presence of *Leptospira* spp. in bovine kidney and to determine its predominant species. Moreover, to investigate the associated lesions in kidney tissues from selected wet markets in this region, particularly the state of Kelantan. Various diagnostic methods were used to detect the *Leptospira* spp., including molecular detection via polymerase chain reaction (PCR) and nucleotide sequencing analysis. Lastly, histopathological examination was carried out to evaluate kidney lesions attribute to BL.

2.0 Research problem

Various studies have been carried out on *Leptospira* spp. detection in Malaysia, but mostly involved the serological detection from the serum samples in live animals. However, the reports of detection of the organism from the bovine carcass, particularly in the kidney tissue is not documented in Kelantan, Malaysia.

3.0 Research questions

3.1 Does bovine kidneys infected with *Leptospira* spp. sold in wet markets in Kota Bharu, Kelantan?

3.2 What are the common *Leptospira* spp. that can be identified from bovine kidneys in Kota Bharu, Kelantan, Malaysia?

3.3 What are the histopathological lesions can be investigated from the bovine kidneys infected by leptospirosis?

4.0 Research hypothesis

4.1 There is presence of *Leptospira* spp. in the bovine kidneys sold in wet markets in Kota Bharu, Kelantan.

4.2 The common *Leptospira* spp. that can be detected from bovine kidney are *Leptospira interrogans* and *Leptospira borgpetersenii*.

4.3 The histopathological lesions related to bovine kidney infected by leptospirosis are renal tubular necrosis, interstitial nephritis and interstitial fibrosis.

5.0 Objectives

5.1 To detect the *Leptospira* spp. in the bovine kidneys sold in selected wet markets in Kota Bharu, Kelantan.

5.2 To determine the species of *Leptospira* in the bovine kidneys sold in wet markets in Kota Bharu, Kelantan.

5.3 To investigate the histopathological lesions in bovine kidney tissues infected by leptospirosis.

6.0 Literature review

6.1 Description of *Leptospira* spp.

Leptospira is a genus of spirochaete bacteria from the family *Leptospiraceae*, which is a pathogen of veterinary and human medical importance. *Leptospira* spp. are long, thin, helical bacteria with hook-shaped ends and the size range from 0.1 x 6μm to 12μm (Quinn et al., 2011). The organism has an inner membrane wrapped around a straight central axial filament which contain a longitude periplasmic flagella exiting the cell in subterminal locations. Due to this feature, *Leptospira* spp. are motile, which moves in a spiral movement and rotating along the long axis. Although *Leptospira* spp. is Gram negative bacteria, however, they are poorly stained with conventional bacteriological dyes and commonly visualized using dark field microscopy as well as silver impregnation and other immunological staining techniques (Greene, 2006).

Leptospira spp. can survive in various water sources such as ponds, moist soil and mud when environmental temperatures are neither high or low (between 0˚C to 25˚C) and reduce viability if expose with freezing, dehydration and harsh sunlight radiation (Greene, 2006; Levett & Haake, 2010). Stagnant or slow-moving warm water provides a favourable environment for this bacteria. *Leptospira* spp. can form biofilms on both organic and inorganic surfaces within those aquatic environments. In addition, this bacteria can survive in soil with neutral or slightly alkaline pH (Greene, 2006).

6.2 Bovine Leptospirosis (BL)

Leptospirosis is a zoonotic disease, which pathogenic to both humans and animals. This disease occurs worldwide and is endemic in countries with humid and tropical climates. Studies estimate that there are more than 500,000 cases of leptospirosis each year. These cases have been reported in countries of Central and South America, Southeast Asia as well as Oceania (Pappas et al., 2008).

In Malaysia, leptospirosis is endemic, which the emergence has become a significant human and veterinary public health concerns. *Leptospira* spp. can establish infections in a variety animal hosts, including domestic animals, which can cause an occupational hazard for people who work with animals. Cattle is natural maintenance hosts for leptospira which retain the bacteria chronically in the kidney. Principally, cattle are the maintenance host for *L. borgpetersenii* serovar Hardjo as well as *L. interrogans* serovar Harjo, which can accidentally affect small ruminants and human (Radostits, et al., 2006). Table 6.1 shows the common leptospiral species and serovars in cattle.

In Malaysia, BL is underreported therefore the current prevalence of BL is not known. One study about bovine leptospirosis was carried out in North-eastern Malaysia, which showed an overall prevalence of leptospirosis seropositivity among cattle was 81.7% (Daud, et al., 2018). Though many infections resulted in silent clinical manifestation, infection with non-native species may resulted in severe reproductive disorders such as abortion and still birth, as well reduce

milk production which both directly cause economic loss (Abdul Rahman et al., 2021).

BL can be transmitted to the naïve susceptible cattle and human either through direct or indirect contact with the bodily fluids (urine, blood, semen and milk) and the tissues (aborted fetus, placenta) of the infected animals (Levett & Haake, 2010). The major transmission route that have been identified for leptospiral infection in cattle are open grazing, which the susceptible host accesses to the contaminated water sources (Rajala et al., 2017). Moreover, direct transmission in cattle herd can also occurred through the venereal transmission of natural service (Loureiro et al., 2017) and vertical transmission from the infected cows to the calves (Aqib et al., 2019).

Human can obtain the bovine leptospiral disease by occupational exposure, such as transmission to the veterinarians, workers in the farms, abattoir and milking sheds as well as the butchers (Zarantonelli et al., 2018). From the study by Rajala et al (2017), transmission of bovine leptospirosis to human involves the direct contact with the bodily fluids of the infected cattle, especially the milk.

Table 6.1: Common species and serovars of leptospira in cattle

Genotypic classification	Species	Serovars
Pathogenic	*L. interrogans*	Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Harjo-prajitno, Pyrogenes
	L. borgpetersenii	Ballum, Hardjo-bovis, Javanica
	L. noguchii	Pomona, Panama
	L. kirschneri	Grippotyphosa, Cynopteri, Bulgarica
Non-pathogenic (saprophytic)	*L. wolbachii*	Codice
	L. biflexa	Patoc

6.3 Laboratory Diagnosis of Bovine Leptospirosis

Laboratory routine diagnosis of BL can be divided into direct and indirect detection assays. Direct detection of leptospira involves the visualization in blood or urine by darkfield microscope and through a range of staining methods, such as immunofluorescence, immunoperoxidase and silver stains (Agudelo-Flórez et al., 2013). PCR assays targeting several genes also have been developed as one of the direct laboratory detection methods of leptospires (Patricia et al., 2014; Azali et al., 2016). Leptospires can be isolated from the samples by inoculating on a selective media such as Ellinghausen McCullough, Johnson and Harris (EMJH) supplied with an enrichment media (Zuerner, 2006) and customised laboratory media (Guedes et al., 2020). Although leptospiral isolation and culture are considered gold standard for definitive diagnosis in clinical cases, the growth is time consuming and therefore not recommended as diagnostic tool in acute infection.

Indirect detection methods include serological tests, such as microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). Both of these tests are used to detect the presence of antigen-antibody reaction of the serum samples (Levett, 2004; Jayasundara et al,. 2021). Table 6.2 shows the summary of laboratory diagnosis of leptospirosis.

Table 6.2: Laboratory Diagnosis of Leptospirosis

Direct Detection Methods	**References**
Darkfield microscopic examination	Levett & Haake (2010)
Immunohistochemical stain	Almeida et al., (2019)
Silver stain	Agudelo-Flórez, et al., (2013)
Polymerase chain reaction	Vedhagiri K et al., (2010)
Bacterial culture and isolation	Guedes et al., (2020)
Indirect Detection Methods	
Microscopic agglutination test	Daud et al., (2018)
Enzyme-linked immunosorbent assay	Surujballi & Mallory (2004)

6.4 Histopathological Changes of the Infected Kidney

Following infection, the organisms can spread via hematogenous route and further replicate in the organs, including liver and kidney. Antibody titre increases as early as seven to eight days after infection, which will clear the *Leptospira* spp. from the blood and most organs, but the organisms may persist in the kidney and shed in urine for days to months (Greene, 2006). Based on the study by Monahan, Callanan & Nally (2009), the mechanism of immune evasion by *Leptospira* spp. during renal colonization is possibly due to the absence of immune complements.

Histopathological examination can be carried out by using the infected organs, which will be fixed in neutral-buffered formalin and embedded in paraffin. All the tissue sections will then stain and examined under compound microscope to observe the morphological changes. According to the study by Agudelo-Flórez et al (2013), the lesions that normally can be observed in the infected kidneys are interstitial nephritis, perivascular infiltration, glomerulitis, glomerulo-tubular atrophy and hyalinosis that suggestive for kidney injuries as a result of leptospiral colonization and proliferation.

7.0 Materials and methods

7.1 Sample Collection and Preparation

Convenience sampling was carried out by collecting a total of 50 bovine kidney samples (about 20 g each) from four wet markets in Kota Bharu, Kelantan, Malaysia. The samples were kept individually in a sterile seal bag and stored in a polystyrene box with ice packs during transportation. Samples were then stored and froze at -80°C for further analysis. For histopathological examination, eight kidney samples (about 5 g from each sample) which including both showed positive results and negative results for PCR assays, were stored in a 10% (v/v) neutral buffer formalin for 24 hours to fix the sample.

7.2 Molecular Detection of *Leptospira* spp.

7.2.1 DNA Extraction

DNA extraction was performed using a commercial DNA extraction kit (Geneaid, Taiwan) following manufacturer's instruction. Up to 25 mg fresh kidney tissue sample was transferred to a 1.5 ml sterile microcentrifuge tube. 200 µl of GST buffer and 20 µl of proteinase K were added into the tube and vortexed thoroughly. Micropestle was used to grind the tissue sample. The sample lysate was incubated at 60˚C overnight to dissociate the tissue.

After overnight incubation, 200 µl of GSB buffer was added into the sample lysate and was shaked vigorously for 10 seconds to lyse the cells. For DNA

binding, 200 µl of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously for 10 seconds. GS column was placed in a 2 ml collection tube. All of the mixture was transferred to the GS column and was centrifuges at 14,000 x g for 5 minutes. Following centrifugation, the 2 ml collection tube containing the flow-through was discarded and the GS column was transferred to a new collection tube.

To wash the DNA pellet, 400 µl W1 buffer was added to the GS column and was centrifuged at 14,000 x g for 1 minute. The flow-through was then discarded and the GS column was placed back into the 2 ml collection tube. 600 µl of wash buffer with absolute ethanol was added to the GS column. The tube was centrifuged at 14,000 x g for 1 minute and the flow-through was discarded. GS column was placed back into the 2 ml collection tube and was centrifuged at 14,000 x g for 5 minutes to dry the column matrix.

The dried column was transferred to a new sterile 1.5 ml microcentrifuge tube. 100 µl of pre-heated elution buffer was added into the center of the column matrix and was allowed to stand for 3 minutes. The tube was centrifuged at 14,000 x g for 2 minutes to elute the purified DNA. Purified DNA was then stored in a freezer at -20˚C.

7.2.2 Primer Sequence

Two sets of primers were used in this study for PCR to detect the presence of *Leptospira* spp., DNA in bovine kidney tissue samples. Table 7.1 shows the list of primers used in this study.

Table 7.1: The list of primers used in this study

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

7.2.3 DNA Amplification by PCR

The molecular detection of *Leptospira* spp. was performed by 16S rRNA and LipL32 gene amplification using the specific primers listed in the Table 7.1. 15 µl of PCR reaction mixture contained 12.5 µl PCR master mix, 1 µl of 10 mM forward primer, 1 µl of 10 mM reverse primer and 0.5 µl nuclease free water was added into 0.2 ml PCR tube. About 10 µl DNA sample was then added to the tube. The 16S rRNA and LipL32 fragments were amplified by using an Eppendorf thermal cycler. The condition of each PCR reaction was carried out using the published studies (Table 7.1) with slight modifications. Positive (*L. interrogans*) and negative (nuclease free water) controls were included for each cycle.

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 minutes and final elongation at 72˚C for 5 minutes. LipL32 gene were then 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.

7.2.4 PCR Product Detection

The amplified products were evaluated by agarose gel electrophoresis. 2% (w/v) agarose gel was prepared 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. 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 GelDoc™ 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.

7.3 DNA Purification

DNA purification was carried out by using commercial purification kit (Geneaid, Taiwan). The agarose gel slice containing relevant DNA fragments were excised and the extra agarose was removed to minimize the size of gel slice using a commercial gel extraction protocol with manufacturer's instruction. Briefly, up to 300 mg of the gel slice was transferred to a 1.5 ml microcentrifuge tube. 500 µl of DF buffer was transferred to the tube and was mixed by vortex. The tube was then incubated at 60˚C for 15 minutes until the gel slice had completely dissolved. The dissolved sample mixture was allowed to cool down to room temperature.

The DF column was placed in a 2 ml collection tube. For DNA binding, 800 µl of the sample mixture was transferred to the DF column and was centrifuged at 15,000 x g for 1 minute. The flow-through was discarded and the DF column was placed back in the 2 ml collection tube.

To wash the DNA pellet, 600 µl of wash buffer with ethanol was added into the DF column and was allowed to stand for 1 minute. After 1 minute, the tube was centrifuged at 15,000 x g for 1 minute. The flow-through was discarded and the DF column was placed back in the 2 ml collection tube. The steps were repeated to wash the DNA pellet. The tube was centrifuged again at 15,000 x g for 3 minutes to dry the column matrix.

The dried column matrix was transferred to a 1.5 ml microcentrifuge tube. 50 µl elution buffer was added into the centre of the column matrix and was

allowed to stand for at least 2 minutes. The tube was centrifuged at 15,000 x g for 2 minutes to elute the purified DNA. The purified DNA was then sent to Apical Scientific SDN BHD for DNA Sanger Sequencing.

7.4 Histopathological Examination

Eight bovine kidney samples that including those showed positive and negative results in molecular detection of *Leptopsira* spp. were trimmed and fixed in 10% (v/v) formaldehyde for 24 hours. The fixed specimens were stained with haematoxylin and eosin (H&E) stain and were observed under compound microscope to evaluate the histopathological lesions. The lesions were classified according to the histopathology scoring system by a study by Prakoso, Widyarini & Kurniasih (2020) which showed in Table 7.2.

Table 7.2: Histopathology scoring system used for kidney evaluation

Score	Hematoxylin & Eosin			
	Severity	**Duration**	**Distribution**	**Exudate**
0	NHC	NHC	NHC	NHC
1	Minimal	Acute	Focal	Suppurative
2	Mild	Chronic	Multifocal	Fibrinous
3	Moderate	Chronic active	Locally extensive	Necrotizing
4	Severe	-	Diffuse	Fibrinopurulent
5	-	-	-	Granulomatous

NHC: No histopathological changes

7.5 Statistical Analysis

The result was recorded and statistically analysed by using manual record and tabulation by Microsoft Office to detect the *Leptospira* spp. in bovine kidney and to investigate the histopathological lesions in the infected kidney.

8.0 Results

A total 50 bovine kidney samples were collected from four wet markets in Kota Bharu, Kelantan. Amplification of the 16S rRNA of *Leptospira* spp. showed the presence of amplicons at around 330 bp (Figure 8.1). Six out of 50 samples (Sample no 18, 19, 20, 22, 24 and 25) collected were detected positive which four samples were collected from wet market A and two from wet market B (Table 8.1). The other 44 were found to be negative. On the other hand, amplification of the LipL32 gene of *Leptospira* spp. showed the presence of amplicons at around 500 bp (Figure 8.2), which differs from the previous study which the size was at about 700 bp. One out of 50 samples (Sample 22) was detected positive with the presence of LipL32 gene of *Leptospira* spp., which were collected from wet market A. Due to poor amplification, only two purified DNA samples that positive for 16S rRNA PCR (sample no 18 and 22) were successfully submitted for sequencing. The obtained sequences were compared using the BLAST program against the GenBank database. The identical organisms were showed in Table 8.2 based on the top five line of BLAST database sequence. From the sequencing analysis, both samples were shown to be 99% identical to *L. borgpetersenii*.

Table 8.1: Results of PCR in bovine kidney tissue samples collected from wet markets

Wet market	No. of samples	No. of positive	
		16S rRNA	LipL32
A	23	4	1
B	16	2	0
C	7	0	0
D	4	0	0
Total	50	0	0
Percentage		**12%**	**2%**

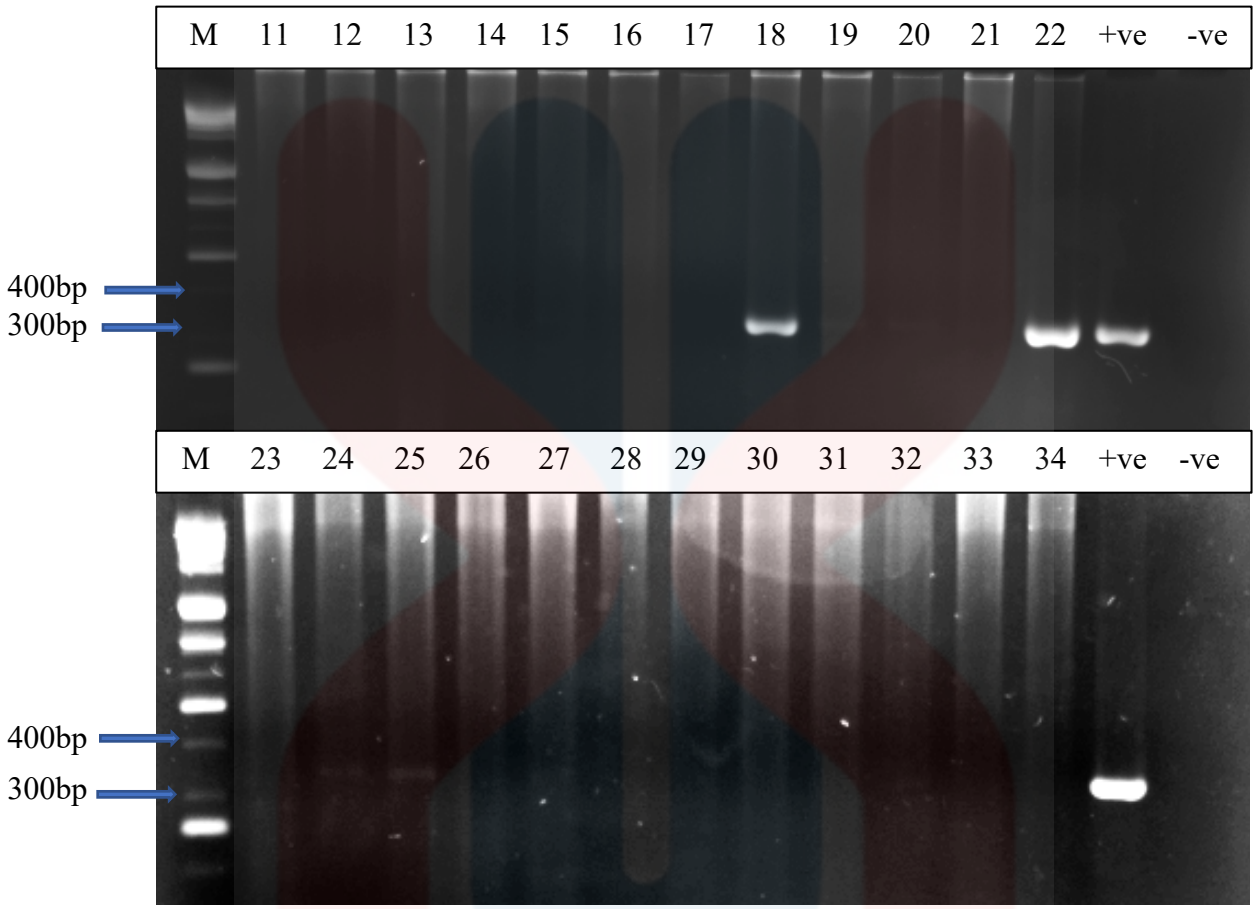

Figure 8.1: PCR amplification of 16S rRNA gene of Letospira spp. demonstrated by agarose gel electrophoresis. M: 100bp ladder; lane 2 to 13: PCR products; +ve: positive control; -ve: negative control.

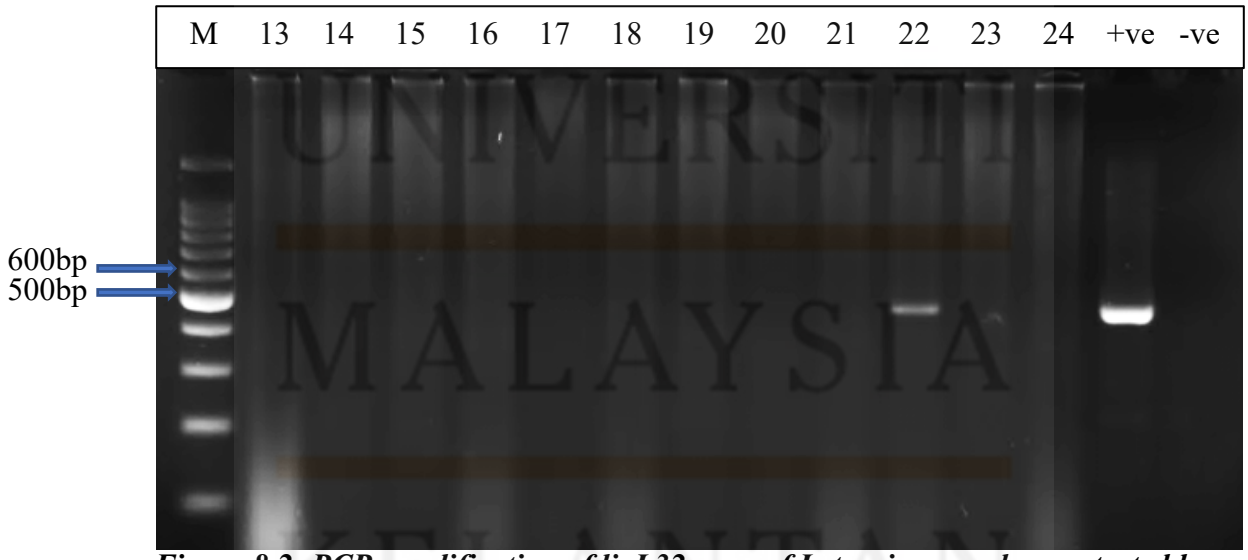

Figure 8.2: PCR amplification of lipL32 gene of Letospira spp. demonstrated by agarose gel electrophoresis. M: 100bp ladder; lane 2 to 13: PCR products; +ve: positive control; -ve: negative control.

Table 8.2: DNA sequencing analysis of Leptospira spp. PCR assay products.

Samples	Identical organisms	Max score	Total score	Query score	E-value	Percentage ID	Accession No.
18	*Leptospira borgpetersenii* strain R6L chromosome 1	536	536	99%	4e-151	99%	CP047520.1
	Leptospira borgpetersenii strain R14 chromosome 1	536	536	99%	4e-151	99%	CP047504.1
	Leptospira borgpetersenii strain R6 chromosome 1	536	536	99%	4e-151	99%	CP047372.1
	Leptospira borgpetersenii strain Mo4 chromosome 1	536	536	99%	4e-151	99%	CP047334.1
	Leptospira borgpetersenii strain R28 chromosome 1	536	536	99%	4e-151	99%	CP047332.1
	Leptospira borgpetersenii strain R6L chromosome 1	540	540	96%	3e-152	100%	CP047520.1
22	*Leptospira borgpetersenii* strain R14 chromosome 1	540	540	96%	3e-152	100%	CP047504.1
	Leptospira borgpetersenii strain R6 chromosome 1	540	540	96%	3e-152	100%	CP047372.1
	Leptospira borgpetersenii strain Mo4 chromosome 1	540	540	96%	3e-152	100%	CP047334.1
	Leptospira borgpetersenii strain R28 chromosome 1	540	540	96%	3e-152	100%	CP047332.1
	Leptospira borgpetersenii strain R23 chromosome 1	540	540	96%	3e-152	100%	CP047370.1

The bovine kidney samples collected from four wet markets did not have evident of macroscopic or obvious gross lesions towards presence of leptospira. Eight samples, which included the samples detected positive upon PCR detection were selected for histopathological examination as described in methodology section. All eight kidney samples showed morphological changes and the lesions were classified according to the histopathology scoring system which showed in Table 7.2.

The hematoxylin-eosin score showed the severity of the histopathological lesions, which the higher score indicated the increasing in severity of the lesions (Table 8.3). All of samples showed severe diffuse necrotizing nephritis, regardless positive and negative upon PCR detection. Sample no 22 which was positive for both 16S rRNA and LipL32 gene detection, had the highest hematoxylin-eosin score and showed severe chronic active diffuse necrotizing tubulointerstitial nephritis which characterised by interstitial infiltration of inflammatory cells, perivascular cuffing hyaline degeneration and tubular necrosis (Figure 8.3). The remaining samples showed mixed histopathological lesions consisting of severe chronic diffuse necrotizing tubulointerstitial nephritis (sample 25, 32 and 35) and severe diffuse necrotizing glomerulonephritis (sample no 3, 11, 18 and 45), respectively.

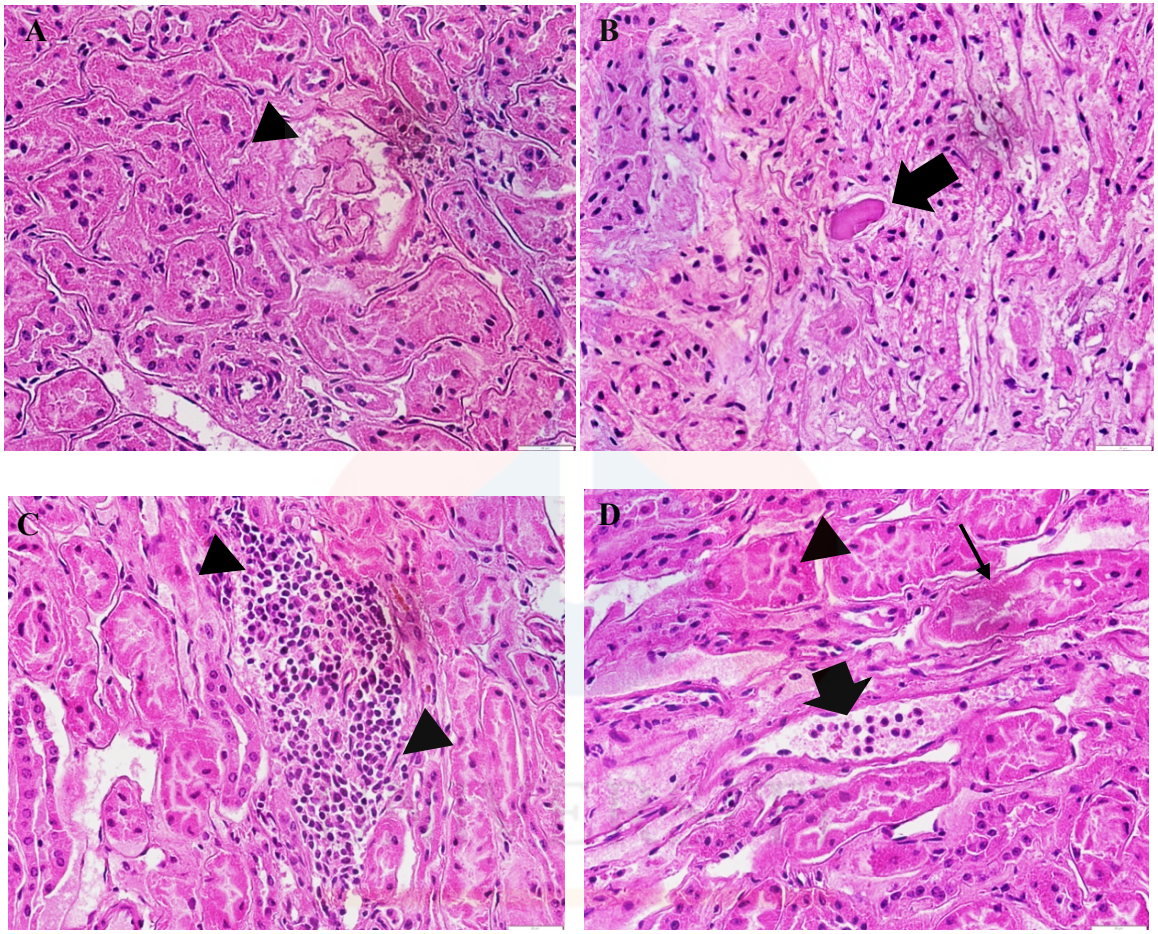

Figure 8.3: Histopathological changes in No. 22 bovine kidney tissue samples; H&E stain (magnification of 400x). A: glomerular atrophy (arrow head). B: deposition of pinkish homogenous hyaline(arrow). C: infiltration of inflammatory cells within the interstitial (arrow head). D: eosinophilic droplets in the tubular epithelial cells (arrow head), tubular necrosis (thin arrow) and margination of inflammatory cells in the lumen (thick arrow).

Table 8.3: Histopathology scoring of bovine kidney samples

Kidney	PCR-16S rRNA	PCR-LipL32	Morphological diagnosis	H&E score
3	-	-	Severe diffuse necrotizing glomerulonephritis	11
11	-	-	Severe diffuse necrotizing glomerulonephritis	11
18	+	-	Severe diffuse necrotizing glomerulonephritis	11
22	+	+	Severe chronic active diffuse necrotizing tubulointerstitial nephritis	14
25	+	-	Severe chronic diffuse necrotizing tubulointerstitial nephritis	13
32	-	-	Severe chronic diffuse necrotizing tubulointerstitial nephritis	13
35	-	-	Severe chronic diffuse necrotizing tubulointerstitial nephritis	13
45	-	-	Severe diffuse necrotizing glomerulonephritis	11

+: positive, -: negative

9.0 Discussion

Bovine leptospirosis caused by different species of leptospira, is a widespread zoonotic disease. Livestock farming and abattoir that are associated with beef or milk production are the major occupational risk factor for bovine leptospirosis throughout the world, including in Malaysia (Morey et al., 2006). In order to figure out the potential zoonotic risk, a study was carried out to detect the *Leptospira* spp. in bovine kidney samples.

This was the first study in Kelantan that successfully detect *Leptospira* spp. in bovine kidney samples. Molecular detection, using PCR provides rapid results in contrast to other time consuming methods and it is also used as confirmatory diagnostic test due to the higher sensitivities and specificities. PCR has been extensively used to detect the presence of leptospiral DNA by targeting certain genes such as universal 16S rRNA and the other surface proteins on the species, such as OmpL1, LipL32, LipL36 and LipL41 (Gokmen, 2016). In this study, two primers were used including the 16S rRNA and LipL32 gene. All type of leptospiral strains, including pathogenic, intermediate and non-pathogenic have 16S ribosomal RNA subunit, which can be identified by molecular detection of 16S rRNA gene (Morey et al., 2006). LipL32 gene is a 32 kDa surface lipoprotein that is conserved in pathogenic leptospiral species, but absent in non-pathogenic and intermediate types (Haake et al., 2000). This can be used to detect pathogenic *Leptospira* spp. using samples such as tissues, blood and bodily discharges such as urine, semen and vaginal fluid from animals.

Out of 50 bovine kidney samples collected, only six samples showed positive for detection of 16S rRNA gene, while one samples showed positive for detection of LipL32 gene. Due to poor yield, only two samples were successfully submitted for sequencing which both revealed to be *L. borgpetersenii.* Interestingly, the Lipl32 product which supposed to yield 700 bp from the previous study (Chaemchuen et al., 2011), was found about 500 bp in this study for both sample no 22 and positive control. We could not explain this anomaly, because the sequencing result for sample no 22 of LipL32 gene identified as *L. borgpetersenii.* We believe there is a gene deletion upon amplification which resulted in reduction of product size. This warrants further investigation.

Additionally, sample no 18 that was positive upon 16S rRNA PCR, was found to be somehow negative upon LipL32. We have repeated the assay twice and as a result a faint band was produced (image not shown) and it was impossible to purify and to submit for further sequencing. This can be due to the low DNA concentration loaded on the gel. Four kidney samples (sample no 19, 20, 24 and 25) which was positive on 16S rRNA PCR may carry either non-pathogenic or intermediate strains of the *Leptospira* spp. We however, could not provide the molecular evidence for this as the PCR yield was beyond poor and therefore could not be submitted for sequencing.

In Malaysia, there were 38 leptospiral serovars that had been isolated from animals (Bahaman, Ibrahim & Adam, 1987). Bovine leptospirosis can be caused by a variety of pathogenic leptospiral serovars. According to Levett (2004), most cases in livestock, including bovine occurred subclinical or showing mild clinical signs. Detection of the leptospiral serovars is highly recommended in diagnosis of bovine leptospirosis and also important for epidemiological perspectives. Based on the previous study by Khairani et al., (2004), *L. borgpetersenii* serovar Hardjo-bovis is a common serovar that is maintained by cattle, which most of the infected animals are apparently healthy. In this study, two samples were found to carry *L. borgpetersenii*. Though the number is small, this shows that the cattle in Kelantan potentially carry the bacteria and able to shed it via urine which may infect human and other animals. *L. borgpetersenii* is one of the pathogenic species of *Leptospira* which has limited survival capability compared to its closely related pathogenic species such as *L. interrogans* due to its genome reduction that affect its ability to survive out the host (Bulach et al., 2006). The cattle may get the infection (with or without clinical signs) via various possibilities, such as direct contact with other cattle in the herd (Levett & Haake, 2010), venereal transmission (Loureiro et al., 2017), maternal transmission during pregnancy or suckling (Aqib et al., 2019). We could not determine the type of serovars under *L. borgpetersenii* using our samples. Therefore, serum samples should be collected prior animal slaughter to determine the serovars via MAT.

According to the study by Levett & Haake (2010), leptospiral renal colonization occurred in the infected hosts at the leptospiruria phase, which the bacteria will be shed in the urine. The presence of leptospiral bacteria can cause direct damage or indirect damage to the kidney due to the leptospiral antigen initiated immune response, resulting in certain histopathological alteration. In the presence study,

histopathological alterations can be observed in the kidney infected by leptospiral bacteria was characterized as chronic, with the infiltration of inflammatory cells that was composed predominantly of lymphoplasmocytic cells (Carvalho, et al., 2011).

In Malaysia, the study related to leptospiral renal colonization and histopathological alteration was very limited. Thus, in this study, the bovine kidney samples that showed positive results toward the molecular detection were used to perform histopathological examination. The histological alterations observed in the bovine kidney samples are inflammatory cells infiltration, tubular necrosis, glomerular atrophy and hyaline degeneration. All of the samples sent for histopathological examination presented changes suggestive leptospirosis, including those samples that presented negative results upon molecular detection. However, the histological lesions that can be visualized by H&E stain may be non-specific for leptospirosis (Baskervile, 1986). Other diseases such as bacterial pyelonephritis (Divers, 2008) can cause kidney damage, resulting in the similar histopathological changes as leptospirosis. Therefore, additional assays such as immunohistochemistry staining, can be carried out to support the detection and visualize the alterations specific for bovine leptospirosis (Silva et al., 2005).

Based on the study, the results showed the presence of pathogenic leptospiral in bovine kidney tissue samples. This indicates that bovine leptospirosis is maintained in the nature by chronic renal colonization of the maintenance host. Transmission to the human can be occurred through direct or indirect contact with the urine or the kidney tissue samples of the infected animals (Levett & Haake, 2010). This possesses an occupational hazard to the butchers, workers in the wet markets, abattoir and farm workers.

10.0 Conclusion

In conclusion, *L. borgpetersenii* are detected in the bovine kidney samples collected from the wet markets in Kota Bharu, Kelantan, which showed related renal histopathological changes. The detection of pathogenic *Leptospira* spp. in this study revealed the presence of occupational risk and zoonotic risk to the people who worked with the animals as well as who worked in or visited to the wet market.

11.0 Recommendations and future work

For the future study, the sample size should increase using relevant sample size determination to produce meaningful results, which can be used to calculate the prevalence of bovine leptospirosis in Kelantan. Secondly, the study can be enhanced by including other type of samples, such as urine, blood and other body secretions, which are important in studying the pathophysiology of the bovine leptospirosis as well as the stages of leptospiraemia and leptospiruria. Leptospiral isolation and cultivation directly from urine samples are recommended as results from this can be used in epidemiological aspects. In addition, the use of silver stain and immunohistochemical stain maybe helpful in investigating the pathological changes of the kidneys associated with bovine leptospirosis. Other organ samples, such as liver can also be collected as samples to observe the pathological alterations cause by leptospiraemia. Lastly, samples from the environment, such as stagnant water and moist soil around the wet market as well as the samples from people who work with animals, such as farmers, veterinarians and butchers can be collected to estimate the risk of occupational hazard and zoonotic potential of bovine leptospirosis.

Appendix A

Appendix A.1: Bovine kidney sampling from wet market A

Appendix A.2: Primary packaging of the bovine kidney tissue samples

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