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PATHOGENICITY OF *Leptospira icterohaemorrhagiae* serovar *Lai* strain Langkawi
IN GUINEA PIGS *(Cavia porcellus)*

H. Tyagita[1,2], A.R. Bahaman[2*], S. Jasni[3], T.A.T. Ibrahim[2] and N.H. Fuzina[4]

[1] *Universitas Padjadjaran, Jl Raya Bandung, Sumedang KM 21, Jatinangor, Jawa Barat, Indonesia*
[2] *Faculty of Veterinary Medicine, Universiti Putra Malaysia, UPM Serdang, Selangor. Malaysia.*
[3] *Research and Innovation Management Centre, Universiti Malaysia Kelantan, Kota Bharu, Kelantan, Malaysia.*
[4] *Institute of Bioscience, Universiti Putra Malaysia, UPM Serdang, Selangor. Malaysia.*

SUMMARY

A tourist was infected with a new strain of leptospires namely, *Leptospira icterohemorrhagiae* serovar *Lai* strain Langkawi, when he was on vacation in Langkawi, Malaysia. The leptospiral strain was successfully isolated from the patient in the Netherland. In this study, the bacteria were retrieved from Holland and inoculated into fifteen guinea pigs in Universiti Putra Malaysia (UPM) to determine its pathogenicity. The main clinical symptoms in the guinea pigs were decreased appetite and jaundice. Blood profile showed high neutrophil, lymphocyte, PCV, RBC, haemoglobin, leukocyte and thrombocyte counts. Besides that, enhancement of electrolytes such as sodium (Na), chloride (Cl), and potassium (K) was also noted. Biochemical examination showed an increase alkaline phosphatase (ALP), aspartate transaminase (AST) and bilirubin levels. Albumin, alanine transaminase (ALT), blood urea, total protein and creatinine were low values. Histopathological examination under haematoxylin and eosin staining showed evidence of haemorrhages, congestion and oedema in all organs, with inflammatory cell infiltration characterized by neutrophils, lymphocytes and macrophages. Hydropic degeneration and cell necrosis were also common in the findings. Leptospires were detected from Day 2 p.i by silver staining and transmission electron microscopy (TEM). Rise in antibody titre was seen as early as Day 5 p.i and leptospiral DNA was detected by PCR in the kidneys and liver on Day 3 and Day 5, respectively. The findings were indicative of leptospirosis. This study demonstrated that guinea pigs are a suitable animal model to illustrate the clinical symptoms and pathological changes seen following infection with *Leptospira icterohaemorrhagiae* serovar *Lai* strain Langkawi. In general, the symptoms and changes seen in leptospirosis are similar to viral infections and the information and data from this present study would help differentiate infection due to leptospires from that of viral infection. Leptospiral infection has often been misdiagnosed to be viral infection such as influenza and dengue which have similar signs and symptoms as leptospirosis.

Keywords: Leptospira icterohaemorrhagiae, Langkawi strain, pathogenicity, guinea pig,

INTRODUCTION

Leptospirosis is an important zoonosis caused by pathogenic leptospires that are capable of surviving in a wide range of moist environment in tropical countries (Monahan *et al.,* 2009). It is considered to be a global zoonosis. Individuals from developed countries could be exposed through global travel and participation in outdoor recreational activities (Bahaman and Ibrahim, 1988). The clinical manifestation of leptospirosis ranges from mild febrile illness to the icteric-haemorrhages and may be accompanied by severe involvement of internal organs such as the liver, lungs, and kidneys (Bharti, 2003). Leptospirosis often goes unrecognized because of its non-specific presentation and has often been misdiagnosed with other febrile illnesses such as influenza, dengue or malaria (Ko *et al.,* 2009). Leptospirosis in humans apparently is under-reported. The understanding of the epidemiology and pathogenesis of leptospirosis is still lacking (Pappas *et al.,* 2008). There are currently more than 250 recognized serovars of *Leptospira* that are capable to infect humans and animals. In domestic animals, *Leptospira* affect organs causing chronic infection and reproductive disease characterized by abortion, stillbirth and infertility, resulting in significant economic losses (Bharti, 2003). Laboratory animals have been used in infectious disease research. Information obtained on the biochemical and pathological changes in an animal model following infection is important in diagnosis (Olfert and Godson, 2000). Infectious agent invading the body stimulates the immune system initiating biochemical, physiologic and pathologic changes in the infected animal model. Relevant aspects of the pathogenesis have been investigated in both cellular and animal models (Tyagita *et al.,* 2018). The hamsters are the animal model most extensively used to study acute or chronic infection caused by *Leptospira* spp. In this study, the pathogenicity of *Leptospira icterohaemorrhagiae* serovar *Lai* strain Langkawi in guinea pigs as the animal model was examined.

MATERIALS AND METHODS

The Test Bacterial Strain

Leptospira icterohaemorhagiae serovar *Lai* strain Langkawi was isolated from a blood sample of a tourist affected with leptospirosis in a hospital from the Netherland in 2005. The isolate was kindly provided by the Royal Tropical Institute in the Netherlands and

*Corresponding author: Prof. Dato' Dr. Abdul Rani Bahaman (A.R. Bahaman); Telephone: +60122262040; Fax: +60389471971. E-mail: ranibahaman3@gmail.com

cultured in Ellinghausen, McCullough, Johnson and Harris (EMJH) Medium.

Infection of Guinea Pigs and Ethical Statement

In this study, seventeen guinea pigs (*Cavia porcellus*) age 3 weeks of age (weight 250-300 g) were used for the experiment. The animals were divided into 5 groups of three animals each group for experimental infection and one group of two animals as negative controls. After one week acclimatization period, the guinea pigs were inoculated intraperitoneally (i.p) with 0.5 mL low-passage EMJH culture of *L. icterohemorrhagiae* serovar *Lai* strain Langkawi (10^6 cells per ml). Animals acting as negative controls were inoculated i.p with 0.5 mL of sterile EMJH liquid medium. The Animals were observed at least twice a day, and recorded daily for changes in clinical signs. After infection, the guinea pigs were sacrificed serially beginning at Day 1 until Day 7 post-infection (p.i). Blood samples of pre- and post-infection with the *Leptospira* were taken for microscopic agglutination test (MAT). One guinea pig from the negative control group was sacrificed on Day 0 and the other on Day 7 p.i. The animals were anesthetized by an intramuscular (IM) injection of ketamine (30mg/kg) and xylazine (5mg/kg). Protocols for animal experiments were strictly followed according to the guidelines of the Animal Care and Use Committee (ACUC), Universiti Putra Malaysia (UPM). Animal experiments were with the approval of the Animal Care and Use Committee (IACUC), UPM (Reference No. 08R55/9 dated January 2009).

Haematology and Histopathology

Collection of Blood and Tissue Samples

The guinea pig whole blood was collected into EDTA-coated or heparin-coated vacutainer tubes by using intracardiac technique under anaesthesia. Blood samples were collected, and the serum separated by centrifugation at 1000 *g* for 15 min at room temperature and stored under −20°C until being analysed. Guinea pig complete blood counts and haematological changes were obtained using the Cell-Dyn 3200 Hematology Analyzer (Abbott, Illinois, U.S.A.). Blood chemical parameters were obtained from heparinized samples using the Dimension Xpand Plus Automated Chemistry Analyzer (Siemens Medical Diagnostics Solutions, Newark, Delaware, USA).

Light Microscopy

At the time of infection study (Day 0), liver, lungs, kidneys and spleen were removed and fixed in 10% neutral-buffered formalin for 16 hours and then transferred into 70% alcohol. Samples were subsequently processed in an automatic tissue processor (Leica ASP 300, Leica Microsystems, Germany) and were then embedded in paraffin. Four μm thick sections placed on glass slides were stained with haematoxylin and eosin and silver stain, and then covered with a drop of DPX and a coverslip. Sections were examined under a light microscope (Olympus BX51, Tokyo, Japan).

Transmission Electron Microscope (TEM)

Samples of the liver and spleen were washed in a saline solution, diced into 1 mm^3 cubes and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4. Samples were then washed with the same buffer solution as above and post fixed in 1% cacodylate buffered osmium tetroxide. Following washing with similar buffer solution the samples were dehydrated in ascending concentrations of acetone (35%, 50%, 75%, 95%, and 100%). Samples were then infiltrated with equal mixtures of acetone and resin and embedded in 100% resin in beam capsules and polymerized at 60°C in an oven. Blocks were sectioned by using an ultra-microtome (Leica Ultracut, UCT, Germany), ultra thin sections on copper grids were stained with uranyl acetate and lead citrate and then washed twice with double distilled water. Samples were viewed under a transmission electron microscope (LEO 912AB EFTEM, Omega Filtering System, Germany) opening at 80 kV. Differences between the control and infected groups were recorded and photographed.

Serological Test

Serum samples (control and infected) extracted from the 0.5 mL blood following collection by intracardial puncture were tested against *L. icterohemorrhagiae* serovar *Lai* strain Langkawi by the microscopic agglutination test (MAT). Positive result was indicated by microagglutination, while absence of microagglutination was taken as a negative result.

Bacterial Genomic DNA Isolation and Polymerase Chain Reaction (PCR) Assay

Genomic DNA was extracted from the bacteria isolated from the guinea pig kidney and liver tissue samples, according to the manufacturer's instructions of DNA detection kit (QIAamp DNA mini kit, Germany). PCR assay was performed with 16S rRNA gene specific primers as described (Cinco et al., 1981). The primers (FP: 5' CGC TGG CGG CGC GTC TTA AA 3' and RP: 5'AAG GTC CAC ATC GCC ACT T 3') used for amplification were performed in 25 μl reaction mixture with approximately 50 ηg of template DNA. The temperature profile for amplification was as follows: initial denaturation at 95°C for 5 min, annealing at 54°C for 1 min and followed by a final extension at 72°C for 5 min. All PCR process was subjected to 35 cycles. The amplified products were separated on 12% agarose gel stained with ETBr and visualised in a gel documentation system (Bio-Rad, USA).

RESULTS

The guinea pigs were infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi. Two guinea pigs died on Day 5 and Day 7 p.i. respectively. Table 1 illustrated the haematological changes in the

guinea pigs which showed significant increase in total white blood cell count (neutrophils and lymphocytes) on Day 5 p.i. However, the value of these cells decreased on Day 7 p.i (Table 1). Automated differential complete blood counts were carry out on all blood samples obtained at Day 1, 2, 3, 5, 7 p.i. following infection with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi. No significant differences on blood counts were detected between the infected animals and non-infected animals on Day 1.

As for the red blood cell (RBC) and haemoglobin (Hb) values, the values were significantly decreased, on Day 7 p.i ($3.6b \pm 0.6 \times 10^{12}$/L and $84.5a \pm 43.7$ g/L). Packed cell volume (PCV) level remained stable till Day 5 p.i ($0.3a \pm 0.0$) L/L and decreased on Day 7 p.i ($0.2a \pm 0.0$) L/L. Thrombocytopenia was evident on Day 3 p.i ($130.7c \pm 68.7$) $\times 10^{9}$/L, however, data were not available for Day 5 and Day 7 p.i due to the sudden death of one guinea pig on consecutive days (Table 1).

In Table 2, the fluctuation of sodium (Na) and chloride (Cl) levels was first detected on Day 1 p.i and continued until Day 7 p.i for all serum samples examined. The potassium (K) level continuously decreased from as early as Day 3 p.i ($12.1a \pm 9.8$) until Day 5 p.i (4.5 ± 1.3) in all serum samples from the 5 guinea pigs that were tested. Alkaline phosphatase (ALP) level of 4 tested serum samples decreased continuously until Day 7 p.i while total bilirubin (mean 75.3 umol/L) and conjugated bilirubin (51.3 umol/L) values were elevated on Day 5 p.i. The creatinine level increased exponentially as early as Day 1 p.i ($23.0a + 8.7$) until Day 7 p.i ($46.0b + 12.7$) in all serum samples examined. Albumin level declined on Day 5 p.i (mean 19.6 g/L). However, blood urea level showed an increased level continuously until Day 7 p.i.
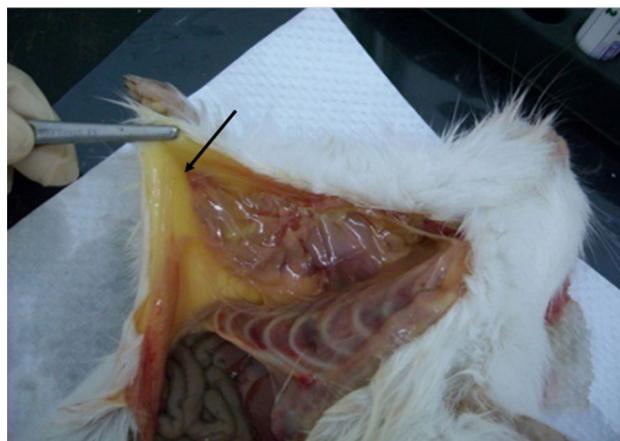

Figure 1. Photograph of an infected guinea pig on Day 5 p.i, showing yellow discoloration of the subcutaneous tissues (arrow)

The infected guinea pigs showed evidence of jaundice on Day 5 until Day 7 p.i. The fat on the skin was discoloured yellow (Figure 1). Haemorrhage was also seen in the *musculus intercostalis,* lungs, liver, intestine, stomach, spleen and kidneys.

Pulmonary congestion was seen in the lungs, followed by alveolar and petechial lobular haemorrhages in the apical, cardiac and diaphragmatic lobes and pulmonary oedema which led to the smaller alveolar lumina, were frequently observed in our study. The alveolar septum was distended due to alveolitis.

Proliferation and pyknosis of pneumocytes Type I and Type II were observed (Figure 5). Perivascular fibrosis, necrosis of bronchioles with necrotic epithelium

Table 1. Haematological values of Negative Control and Guinea Pigs Infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi.

Haematological values	Days				
	1	2	3	5	7
RBC	4.6 ± 0.5^a	$4.2^ab \pm 0.7$	$4.9^a \pm 0.2$	$4.2^a \pm 0.2$	$3.6b \pm 0.6$
Hb	114.0 ± 14.1^a	$103.7^{ab} \pm 6.2$	$115.7^b \pm 17.2$	$102.0^a \pm 0.7$	$84.5^a \pm 43.7$
PCV	$0.3^a \pm 0.0$	$1.0^a \pm 1.1$	$0.3^a \pm 0.0$	0.3^a	$2.2^a \pm 0.0$
WBC	$2.7^a \pm 1.0$	$2.6^{ab} \pm 0.9$	$2.5^a \pm 1.3$	$5.6^b \pm 0.8$	$3.1^a \pm 1.3$
Band neutrophils	0	0	$0.8^a \pm 0.5$	$0.1^b \pm 0.0$	0
Segmented neutrophils	$1.0^a \pm 0.5$	$0.7^{ab} \pm 0.4$	$0.8^a \pm 0.5$	$4.0^b \pm 0.6$	$1.6^a \pm 1.3$
Lymphocytes	$1.5^a \pm 0.4$	$1.6^a \pm 0.6$	$0.8^a \pm 0.5$	$4.0^b \pm 0.6$	$1.6^a \pm 1.3$
Monocytes	0.1^a	$0.1^a \pm 0.1$	$0.3^a \pm 0.2$	$0.3^a \pm 0.2$	$0.2^a \pm 0.0$
Eosinophils	$0.1^a \pm 0.1$	$0.1^a \pm 0.1$	$0.1^a \pm 0.1$	0.1^a	0
Basophils	0	0	0	0	0
Thrombocytes	$378.0^d \pm 38.2$	$322.3^{abcd} \pm 69.3$	$130.7^c \pm 68.7$	$32.0^a \pm 8.5$	$241.0^b \pm 31.1$

^aValues were presented as means $\pm$ SD. Means for each characteristic followed by the same letter within the same column were not significantly different at P<0.05 by LSD test.
The letter superscripts ^{b, c, and d} attached to the means indicate significant difference between 2 means if the means have different superscripts (e.g. ^a and ^b) but not significantly different if the means have the same superscripts (e.g. ^a and ^a).

Table 2. Blood Biochemistry of Negative Control and Guinea Pigs Infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi.

Blood Biochemistry	Days				
	1	2	3	5	7
Albumin	24.5[c] ± 0.2	22.3[ab] ± 2.0	26.0[bcd] ± 0.7	19.6[a] ± 0.6	21.0[a] ± 1.3
ALT	52.6[a] ± 23.4	20.4[a] ± 14.9	24.5[a] ± 3.9	31.3[a] ± 6.4	23.8[a] ± 17.5
ALP	429.0[b] ± 88.6	376.0[ab] ± 68.5	304.3[b] ± 68.0	91.0[a] ± 11.3	80.5[a] ± 44.5
AST	131.9[a] ± 73.7	107.7[a] ± 78.7	59.7[a] ± 11.0	86.[a] ± 32.5	60.3[a] ± 25.8
Direct bilirubin	0.1[a]	0.1[abc]	0.1[a]	51.3[b] ± 26.5	148.3[c] ± 11.0
Total bilirubin	0.2[a] ± 0.2	1.4[abc] ± 2.3	0.4[a] ± 0.3	75.3[b] ± 12.6	178.2 ± 2.6
Calcium	98.0[a] ± 3.1	100.3[ab] ± 5.1	99.8[a] ± 2.5	90.6[b] ± 1.8	97.0[a] ± 6.7
Creatine kinase	1373.0 ± 756.0	335.7[a] ± 114.3	429.7[a] ± 185.3	1227.0 ± 301.2	451[a] ± 346.5
Creatinine	23.0[a] ± 8.7	21.0[ab] ± 5.2	38.0[a] ± 6.1	50.0[b] ± 4.2	46.0[b] ± 12.7
Urea	8.8[a] ± 1.1	9.2[ab] ± 1.5	11.5 ± 0.5	10.1[a] ± 2.4	17.1[b] ± 0.4
Total proteins	40.0[a] ± 2.1	38.0[abc] ± 4.4	45.0[a] ± 1.1	36.2c ± 1.5	42.8[b] ± 1.6
Sodium	134.7[a] ± 0.9	129.4[a] ± 8.9	137.6[a] ± 3.2	127.1[a] ± 0.6	139.2[a] ± 5.2
Potassium	7.3[a] ± 1.1	12.1[a] ± 9.8	5.1[a] ± 0.5	4.5[a] ± 1.3	7.2[a] ± 1.0

[a]Values were presented as means ± SD. Means for each characteristic followed by the same letter within the same column were not significantly different at P<0.05 by LSD test.
The letter superscripts [b, c, and d] attached to the means indicate significant difference between 2 means if the means have different superscripts (e.g. [a] and [b]) but not significantly different if the means have the same superscripts (e.g. [a] and [a]).
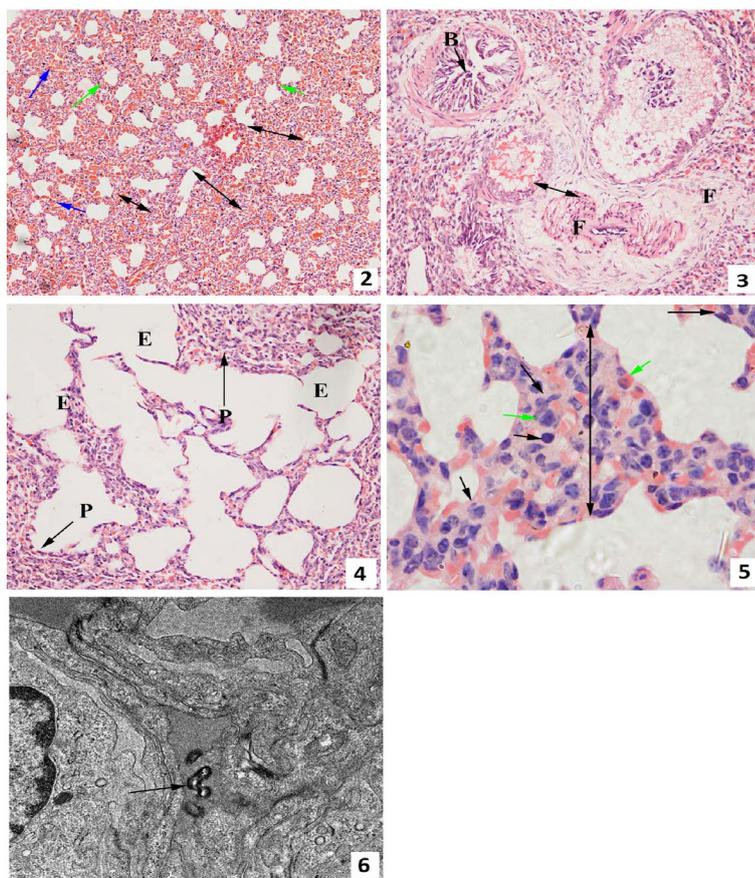

Figure 2. Day 2 p.i, showed a severely thickened interalveolar septa (double arrow) and severe alveolar congestion (blue arrow). The thickened alveolar septae cause smaller alveolar lumina (green arrow). H and E. 100x magnification.

Figure 3. Day 5 p.i, showed a necrotic bronchiole with detachment of necrotic cells epithelium into the bronchiolar lumen (B). Perivascular fibrosis (F) and thickening of the necrotic blood vessel wall is also seen (double arrow). There is also proliferation of tunica media cells in the smaller lumen of the affected blood vessel (double arrow). H and E. 200x magnification.

Figure 4. Photomicrograph of a lung section of a guinea pig sacrificed on Day 5 p.i. Rupture of alveolar septae indicating emphysema (E) is seen. Interstitial pneumonia (P) with infiltration of neutrophils at the interalveolar septa was evident. H and E. 200x magnification.

Figure 5. Lungs of a guinea pig on Day 1 p.i. Note the interalveolar septa (double arrow) are thickened with dilated and congested alveolar capillaries. There is proliferation of pneumocytes Type II (arrow) and scanty infiltration with lymphocytes (green arrow). H and E. 1,000x magnification.

Figure 6. Electron micrograph of a lung section Day 3 p.i, showed leptospires in the alveolar capillaries. Lead citrate and uranyl acetate stain, TEM 3,300x magnification.

detachment into the bronchiolar lumen and hyperplasia of bronchiolar epithelial cells were observed (Figure 3). Severe emphysema, haemorrhages and fibrin infiltration were seen in the lumen of the alveoli (Figure 2 and 4). Presence of *Leptospira* in the lungs could only be seen by TEM (Figure 6).

Severely infected liver was icteric and fragile (Figure 7). Some Kupffer cells were observed to be enlarged and usually seen around the haemorrhagic region. Karyolysis of the hepatocytes and hydropic degeneration of the cytoplasm could also be observed (Figure 8). Congestion of the central vein was an obvious finding in the liver on Day 1 p.i. The congested central veins were surrounded by degenerated and necrotic hepatocytes (Figure 9). The vascular wall in the portal triad area was thickened, filled with pyknosis and

proliferation of tunica media cells (Figure 10). Vasculitis/phlebitis and hyperplasia of bile duct epithelial cells were noted. The hyperplastic cells were detached into the lumen of the bile duct. Occasionally, thrombus can be seen attached to the arterial wall of portal triads (Figure 11). Transmission electron microscopy showed *Leptospira* organisms adjacent and attached to the hepatocyte cell membrane. At the sites of attachment of some *Leptospira* organisms to the hepatocytes with margination of chromatin and some indented nuclei, the cell membrane appeared invaginated as if to accommodate entry of the pathogens into the cell. On Day 5 p.i, some *Leptospira* organisms can be seen in the sinusoids adjacent to hepatocytes (Figure 12).

Figure 7. Photograph of the liver of a guinea pig sacrificed on Day 2 p.i showing yellowish brown liver.

Figure 8. Day 1 p.i. showing hydropic degeneration (D) and karyolysis of hepatocytes (L). Kupffer cells are swollen (K). H and E. 1,000x magnification.

Figure 9. Day 1 p.i. Note central vein congestion (C), severe degeneration and necrosis of hepatocytes (N). The severely swollen hepatocytes (hydropic degeneration) (H) led to loss of sinusoidal architecture. H and E. 400x magnification.

Figure 10. Day 3 p.i, showed necrotic (karyolysis) bile duct (BD), thickening, degeneration (evidence of spaces) (D) and necrosis of arteriolar wall and intimal proliferation (Ar). There are also perivascular haemorrhage (H) and fibrosis (F). H and E. 400x magnification.

Figure 11. Day 5 p.i, showing vasculitis of the vein (phlebitis) and periportal fibrosis (F) and necrosis. Spaces of the inflamed vessel wall were also evident indicating degenerative changes. H and E. 100x magnification.

Figure 12. Electron micrograph of a liver section of a guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain

Langkawi on Day 3 p.i, showing leptospires (arrows) close to hepatocyte. The leptospires are seen closely attached to the cell membrane. At site of adherence, the cell membrane appeared to be invaginated. Lead citrate and uranyl acetate stain. TEM, 2,400x magnification.
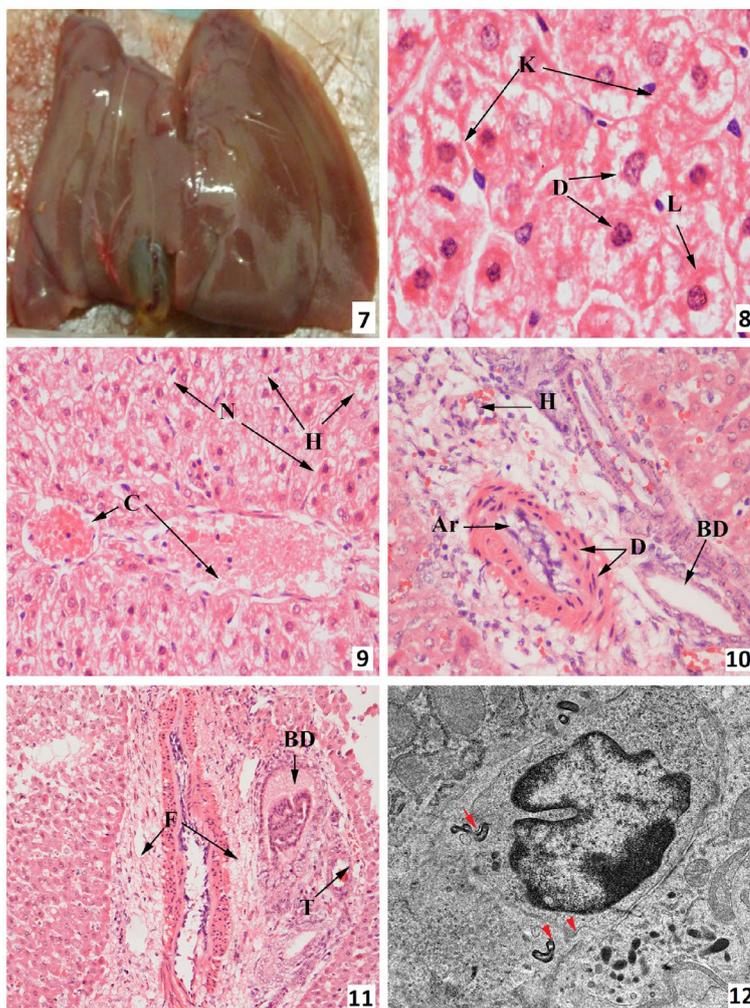
The kidneys were enlarged, soft and the fat of the renal pelvis was yellow indicating icterus. The cortex showed mild whitish streaks (Figure 13). Renal congestion and renal tubule degeneration were observed (Figure 14). Pyknosis of mesangial cells of renal glomeruli was seen. The Bowman's capsule appeared to be ruptured and haemorrhages were observed (Figure 15). At 48 hours p.i, haemorrhages were observed at the renal

cortex especially at the proximal and distal tubules (Figure 16). Atrophy and necrosis of renal corpuscles were more prominent (Figure 17). Lymphocytes were rarely seen but they predominantly focused and infiltrated the interstitium near glomeruli, initially appeared on Day 5 to Day 7 p.i (Figure 18 and 19). Leptospires were detected with silver staining at the interstitium of the collecting ducts.
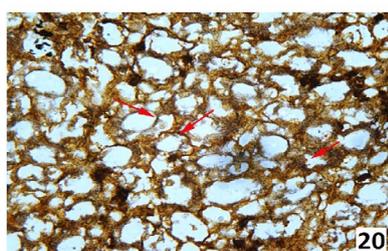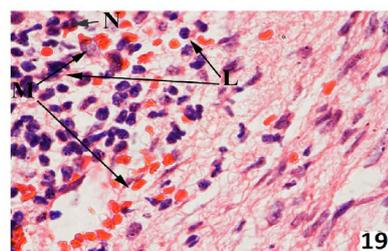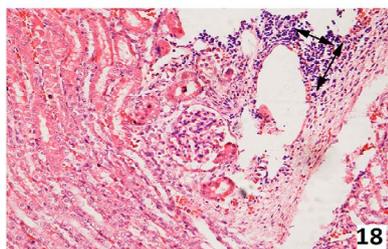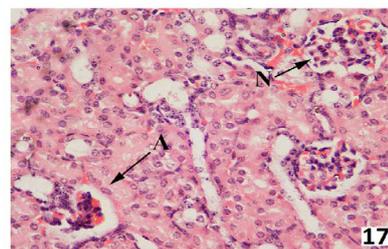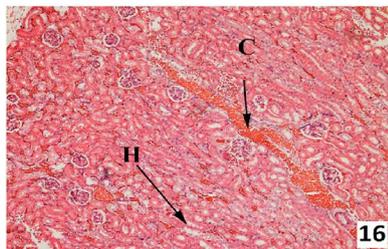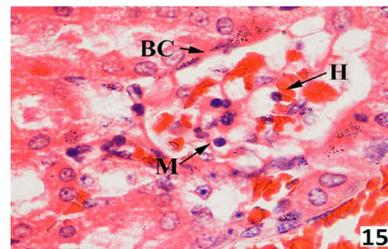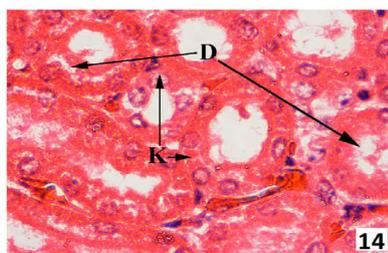
Figure 13. Photograph of a kidney of a guinea pig infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing an enlarged kidney with discoloration of renal pelvis fat. The border between cortex and medulla is congested.

Figure 14. Day 2 p.i, showing karyolysis (K) and hydropic degeneration of tubular cells (D). H and E. 400X magnification.

Figure 15 and 16. Day 2 p.i showing necrotic glomerulus marked by the necrosis of mesangial cell (M). The Bowman's capsule (BC) is ruptured. Evidence of haemorrhages (H). H and E. 1,000X magnification.

Figure 17. Day 3 p.i, showing atrophied (A) and necrotic glomerulus (N). H and E. 400X magnification.

Figure 18. Day 5 p.i. Note focal mononuclear infiltration (interstitial nephritis) (double arrows). H and E. 200X magnification.

Figure 19. Day 5 p.i, higher magnification of Figure 18.
Figure 20. Showing lymphocytes and macrophage infiltration. H and E 1,000X magnification.

In the early-infected stage, the spleen of all the infected guinea pigs were congested (Figure 21) and followed by jaundice (Figure 21a). Pyknosis, proliferation of lymphoid cells and inflammatory cell infiltration of neutrophils, lymphocytes and macrophage were observed, both in the white and red pulps (Figure 22). The white pulp was filled with oedema fluid and red blood cells, replacing the lysed lymphoid follicles. Inflammatory cell infiltration of neutrophils and lymphocytes were prominent (Figure 23). Silver staining detected several leptospires in the sinusoids and intracellularly in the red pulp (Figure 24). TEM showed leptospires in the endothelial cells and membrane cell rupture due to the effect of the bacteria. Swollen rough endoplasmic reticulum, loss of the characteristic cristae of the mitochondria (Figure 25) and necrosis of lymphoid cells were also observed. Other effects such as loss of cellular membrane, disintegration of the rER and cristae of mitochondria (Figure 25) were also observed.

MAT was conducted on all guinea pig sera taken before infection against *Leptospira icterohemorrhagiae* serovar *Lai* strain Langkawi. Leptospiral antibody was not detected in all the sera, thus, indicating no previous history of leptospiral infection. Similarly, serum samples from the infected animals were negative except for two samples. This is expected as antibody develops in later part of infection, after the seventh day of infection.

Seropositivity exhibited by two guinea pigs on Day 5 p.i and Day 7 p.i with titres 1/60 and 1/320 were interesting findings.

PCR assay was performed on tissue samples from the guinea pigs to trace the presence of the leptospires according to organs and time of infection. DNA extracted from 30 tissue samples (15 livers and 15 kidneys) of infected guinea pigs was assayed by PCR and considered positive if there were presence of amplicons (631 bp). In this study, amplicons were seen only in the kidney and liver samples of the guinea pigs that were sacrificed on Day 3 p.i (Lane 11) and Day 7 p.i (Lane 6) respectively. This is an important information where MAT results of guinea pigs sacrificed on Day 3 p.i were negative, whilst DNA of *L. icterohemorrhagiae* serovar *Lai* strain Langkawi was detected in the kidneys by PCR on Day 3 p.i (Figure 25 and 26).

This study was primarily motivated by the lack of information on the pathogenicity of Leptospira on animals and humans. In this study *Leptospira icterohaemorrhagiae* serovar *Lai* strain Langkawi was inoculated into guinea pigs (*Cavia porcellus*) to examine the clinical and pathological responses. This study showed that guinea pigs are a suitable animal model to demonstrate gross and microscopic pictures for leptospiral infection. From the five groups of 15 infected guinea pigs, 2 guinea pigs died, one on Day 5 p.i. and

Figure 20 and 20 a. Photograph of the spleen of negative control, showing a dark red spleen while (B) is a spleen of a guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing bright red and enlarged spleen.
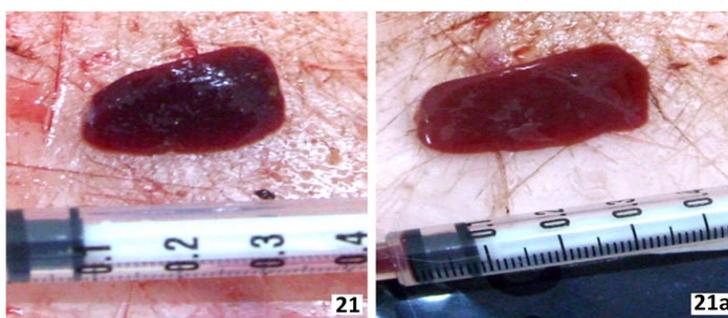
Figure 21. Day 2 p.i, showing infiltration by macrophages (M), neutrophils (N) and lymphocytes (L) in the red pulp. Red blood cells (RBC) were packed in the sinusoid. H and E. 1,000x magnification.
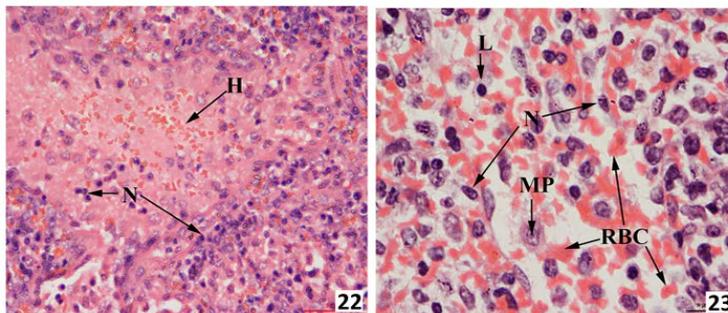
Figure 22. Day 5 p.i, showing a necrotic (karyolysis and pyknosis) white pulp (coagulation necrosis) and infiltration of several neutrophils (N) (splenitis) at these foci that are also haemorrhages (H). H and E. 400x magnification.
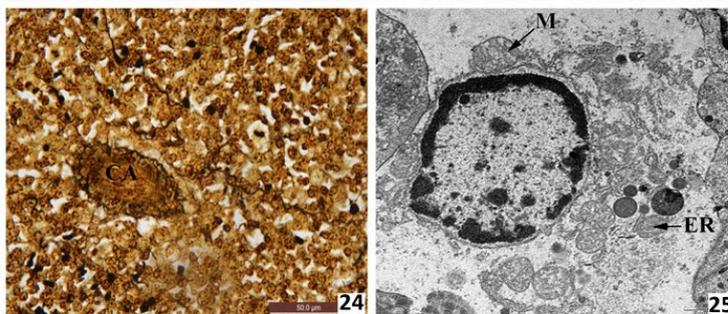
Figure 23. Day 5 p.i, showing leptospires in the central artery (CA) and interstitium of the white pulp. Silver stain. 400x magnification.

Figure 24. Day 5 p.i, showing leptospires in the central artery (CA) and interstitium of the white pulp. Silver stain. 400x magnification.
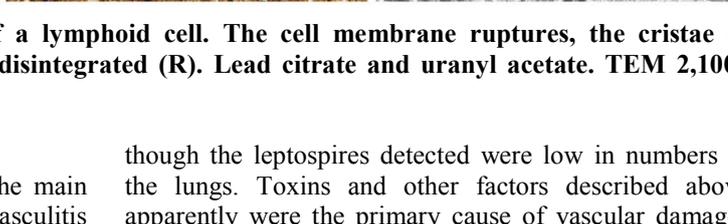
Figure 25. Electron micrograph of a spleen section of a guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing necrosis of a lymphoid cell. The cell membrane ruptures, the cristae of mitochondria disorganized (M) and the rER disintegrated (R). Lead citrate and uranyl acetate. TEM 2,100x magnification.
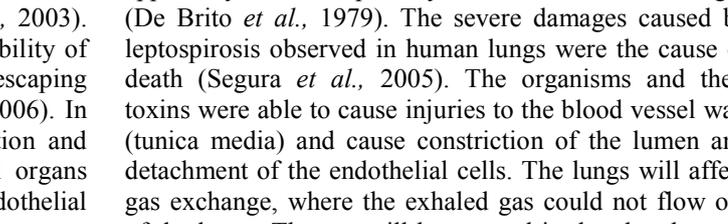
the other on Day 7 p.i.

Leptospiral toxin could be considered as the main damaging factor that resulted in capillary vasculitis (pulmonary haemorrhages) in lungs (Luks *et al.,* 2003). Leptospiral toxin was shown to alter the permeability of sinusoid capillaries resulting in red blood cells escaping and occurrence of haemorrhages (Young *et al.,* 2006). In this study, there was a combination of congestion and oedema in the capillaries and interstitial in all organs examined. These features were due to both endothelial damages caused by leptospires and/or an inflammatory response provoked by leptospiral toxin. Damages on endothelial cell membranes of small blood vessels caused by leptospiral toxin resulted in an instantaneous effect giving rise to the loss of junctions between cells, followed by leakage of proteinaceous fluid and leptospires into extravascular spaces. Erythrocytes were involved in severe and extended damage (De Brito *et al.,* 1979). The von Willebrand factor (vWF), platelet activating Factor (PAF), acetyl hydrolase and paraxonase are known to have the ability to activate the loss of homeostasis (Ren *et al.,* 2003) with two mechanisms of bleeding disorder: (1) direct activation of haemostatic pathways, leading to a consumption coagulopathy and (2) insufficiency autoimmune response stimulation against host haemostatic factors by vWF of leptospiral (Bharti, 2003). However, in this study the findings are consistent even though the leptospires detected were low in numbers in the lungs. Toxins and other factors described above apparently were the primary cause of vascular damages (De Brito *et al.,* 1979). The severe damages caused by leptospirosis observed in human lungs were the cause of death (Segura *et al.,* 2005). The organisms and their toxins were able to cause injuries to the blood vessel wall (tunica media) and cause constriction of the lumen and detachment of the endothelial cells. The lungs will affect gas exchange, where the exhaled gas could not flow out of the lungs. The gas will be trapped in the alveolus and cause emphysema as observed in the guinea pigs starting as early as Day 3 p.i. The emphysema also possibly occurred due to the lesions found in the alveolar wall, where inflammation and necrosis of the interstitial and endothelial cells led to alveolar wall rupture resulting in emphysema.

In the liver, the leptospires were found intracellularly and actively penetrating the neighbouring cells causing detachment of the tight junction resulting in liver cells dissociation. The massive tissue haemorrhages and various degree of hepatocellular necrosis that were seen in our study were also related with occurrence of jaundice (Hartman *et al.,* 1986). Elevation of total bilirubin (mean 75.3 umol/L) and conjugated bilirubin (51.3 umol/L) on the fifth day p.i seen in our study was consistent with the findings (Hartman *et al.,* 1986;

Higgins and Cousineau, 1977). Elevation of these values was related with parenchymal cells of the liver and the spleen that failed in excreting the phagocytosed degraded erythrocytes, resulting in the accumulation of the exhausted erythrocytes in the reticuloendothelial system. The conversion process of haem-bilirubin was impaired and finally the conjugated bilirubin that were converted in the liver, escaped into the blood stream. As a result, bilirubin level was elevated in the blood stream resulting in jaundice (Gancheva *et al.,* 2005). Hyperbilirubinemia in leptospirosis has been reported in some studies (Higgins and Cousineau, 1977). Haemorrhages and congestion were frequent findings in human leptospirosis (Marinho *et al.,* 2009). Haemorrhagic foci and inflammation were observed in the interstitial spaces of kidneys and liver. Infiltration of inflammatory cells such as neutrophils, lymphocytes and macrophages (Marinho *et al.,* 2009). However, jaundice and haemorrhages were not always evident in leptospirosis, but were obviously observed in our study, especially in the peritoneum, lungs, liver, spleen and kidneys (Kobayashi, 2001).

Focal necrosis found in the liver was supported by the TEM findings of necrosis in hepatocytes that affected albumin production. This was related to the liver function in synthesizing albumin. The lesions in liver reduced the functionality and number of hepatic parenchyma cells resulting in diminished albumin production (Sacher *et al.,* 2000). As seen in our study, the albumin level decreased continuously from as early as Day 5 (mean 19,6 g/L) to Day 7 p.i (mean 21.0 g/L). Greene *et al.*, (2013) have reported chronic active hepatitis on Day 5 p.i, marked by fibrosis and mononuclear.
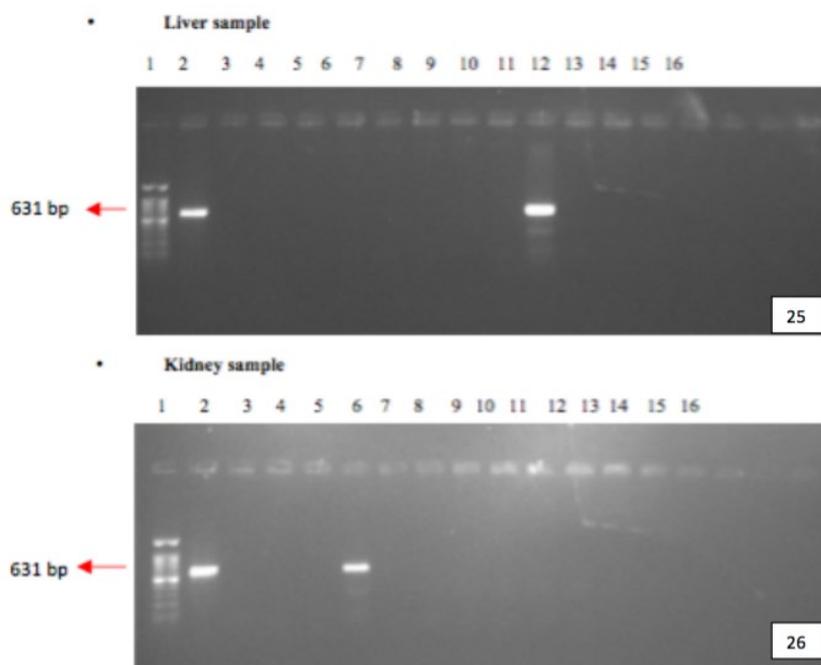

Figure 26 and 27. PCR results of the kidney and liver samples from guinea pigs experimentally Infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi: PCR of kidney (Fig 25) and liver (Fig 26) samples of infected guinea pigs targeting the 16S gene, using the designed primers and visualized in 15% agarose gel. Lane 1. Ladder, Lane 2. Positive control, culture of *L. icterohaemorrhagiae* Serovar Lai Strain Langkawi, Lane 3. Negative Control (dH$_2$O), Lane 4-6. Samples Day 7, Lane 7-9. Samples Day 5, Lane 10-12. Samples Day 3, Lane 13-15. Samples Day 2, Lane 16-18. Samples Day 1.

cell infiltration which then caused altered blood circulation and immunologically mediated injury (Sykes and Greene, 2013). The perivascular fibrosis stimulated the stellate cells resulting in TGF-ß1 secretion, which is potent in fibrotic response such as connective tissue proliferation and ultimately causing blood flow obstruction (Tox and Steffen, 2006). In the present study, hepatitis and fibrosis found in the liver have decreased the ALP level significantly, as early as Day 5 p.i (91.0±11.3) and continued until Day 7 p.i (80.5±44.5). Replacement of active hepatocytes by fibrous connective tissues have been suggested as the causal factor (Center *et al.,* 1995). Even though the sudden appearance of haemorrhagic diathesis and microcirculatory obstruction were observed in this study, it was not enough to prove the presence of disseminated intravascular coagulation (DIC). Decreased thrombocyte count which could start on Day 1 until Day 7 p.i continuously supported the DIC episode because thrombocytopenia is an important factor in determining DIC (Stavitsky, 1945). As seen in this study, fibrinoid-like arrangement with platelets around the blood vessels were due to the body's effort to stop the internal haemorrhages. Together, they formed clots for the ruptured capillaries. As described before, the fibrinoid-like arrangement with platelets and thrombocytes was formed around the injured blood vessels from Day 2 p.i (322.3±69.3) until Day 7 p.i (241.0±31.1) concurrent to the reduction in the diameter of the lumen of blood vessels. The endothelial cell injury reduced prostacyclene synthesis resulting in progressed thrombocytes activation in clotting formation and reduced the number of circulatory platelets. It has been reported that the endotoxin of the leptospires was able to activate the platelets, leucocytes and complement to promote intravascular coagulation (Dolhnikoff *et al.,* 2007) that is responsible for microvascular deposition of fibrin leading to obstructive jaundice (Fletcher *et al.,* 1982). In the kidneys, the lesions were seen in the proximal and distal convoluted tubules, renal corpuscle and also pars recta and collecting tubules. By silver staining, the leptospires were predominantly found in renal collecting tubules. The function of the collecting tubules as collector of the blood filtrate, possibly support the findings. On Day 5 and 7 p.i, the leptospires were detected in the interstitial and in the

lumen of proximal and distal convoluted tubules, pars recta and collecting tubules. This habitat is considered a privileged site where the leptospires are not affected by antibodies or phagocytic cells, they can multiply and then being excreted via urine to contaminate the environment. The results of the current study showed that creatinine and urea levels in the blood were higher than normal (Table1). These affected renal functions marked by tubulonephritis and necrosis of tubular cells and glomerulus that increase tubular creatinine secretion compounded by decreased glomerular filtration rate. As a result, the blood creatinine level was elevated. The lesions also influenced urea waste through the glomerulus. These series of events generated renal azotaemia. Increased urea level concurrent with the enhancement of antibody titre observed in this study, were evidence that the disease has reached the nephritic stage. Tubular cell ischemia seen in our study was possibly related with the bile duct obstruction in the portal triad that facilitated bile salts leakage that could spread to the kidneys. The bile salt infusion would damage aortic endothelium and protective fibrinolytic properties of arterial wall and enhancing intravascular coagulation of the renal arteries (De Brito *et al.,* 1979). The severity of the infection was reduced especially on Day 5 to Day 7 p.i, resulting in hydropic degeneration indicating ischemia or toxic injury or early autolysis especially in the liver and kidneys. Swollen organelles initially occurred, and the cells became water logged, then vacuoles appeared in the cytoplasm and exhibited hydropic degeneration.

In the spleen, the leptospires initially penetrated the capillary wall and were then distributed into the white pulps through central artery and to the red pulps through the sinusoids, as indicated by silver staining. The presence of the leptospires in the white pulps and red pulps resulted in mild to focal necrosis of the area. Haemorrhages of some areas of the white pulps as a result of ruptured vascular wall were observed in our study. This is consistent with findings observed in the spleen of Syrian hamster infected with *Leptospira interrogans* serotype icterohaemorrhagiae (Young *et al.,* 2006). In our study, oedema and congestion were observed in both red and white pulps replacing the necrotic lymphoid cells which make our findings different from other reports. The TEM findings described the ruptured cell membrane and loss of organelles on Day 7 p.i. This is due to attachment of leptospires to the cell wall of lymphocytes. Severe necrosis was observed in the spleen. Being a producer of erythrocytes, damage to the spleen therefore resulted in anaemia. The toxins released by the infected leptospires possibly play a role in causing the anaemia. Other findings such as low PCV, haemoglobin and RBC were considered to be irrelevant. Infection with leptospires has caused severe pathological changes in the lungs and spleen of the guinea pigs in this study. Infection in the kidneys and liver were known to affect major organs resulting in the increase of liver and kidney specific enzymes (Sykes and Greene, 2013). When the disease was in acute stage, haemorrhagic diathesis would be generated and indirectly decrease the liver and kidney functions (Higgins and Cousineau, 1977).

The increase of Na^+, K^+ and Cl^- level in the study were possibly associated with the lesions found in the kidneys caused by the leptospiral endotoxin which was detrimental to the Na^+, K^+ ATPase of the nephron, the molecular target of leptospiral virulence (Khositseth *et al.,* 2008). Fractions of extracted leptospiral glycolipoprotein contain inhibitor for Na^+- K^+ ATPase that affects the apical Na^+, - K^+ and Cl^- cotransporter. By the time dysfunction of Na^+, K^+ and ATPase occurred, it would have caused imbalance of the $Na+$ and $K+$ ion levels. The high Na and Cl level concentration that began on the first day of infection could possibly be affected by limited water intake or water loss from the body. Damage to the kidneys, especially in the distal convoluted tubules and the collecting tubules, which are controlled by aldosterone, would also affect the balance of Na and Cl level. It is believed that it is linked with the disturbance of excretion of K and Cl by the kidneys (Sacher *et al.,* 2000). Increased serum sodium level observed in our findings might also be the cause of excessive water loss of the cells. This would contribute to the occurrence of dehydration and resulting in significantly increased PCV level on Day 7 p.i (2.2 ± 0.0). However, further study is warranted to support this statement. Leucocytes and leucocytes differential count examination were shown to be at a low level on Day 1 until Day 3 p.i. This supported our belief that the infection was still in the leptospiraemia phase at Day 1-3 as the host was attempting to response against the infection, as seen by the increase level of both leucocytes and white blood cell differential count which started on Day 5 p.i. Although the occurrence of leucocytosis was more frequently observed in Weil's disease, leukopenia has also been reported in less severe form of leptospirosis (Mason, 1937).

Specific antibody developed would kill the leptospires and disposed them through the blood stream. However, some of the organisms still exist in the convoluted tubules of the kidneys (Bal *et al.,* 1994). Even though there is bactericidal activity of normal serum, pathogenic *Leptospira* could not be removed without the specific antibodies. So, phagocytosis or destruction by macrophages and polymorphonuclear cells, as observed in the current study, would not function successfully against the organisms (Cinco *et al.,* 1981). The thirteen guinea pigs that survived were sacrificed on Day 1, 2, 3, 5 and 7 p.i. From the two dead guinea pigs supported the PCR results, which showed positive presence of leptospires in the liver on Day 3 p.i and in the kidneys on Day 7 p.i. Silver staining revealed the presence of leptospires in the kidneys as early as Day 2 p.i; liver on Day 3 p.i and spleen on Day 5 p.i. It is seen that the leptospires were distributed in various organs via blood and lymphatic circulation following the intraperitoneal inoculation. In some leptospiral cases, anti-leptospiral antibodies were detected around the seventh day of the illness (Bal *et al.,* 1994). However, in this study using MAT, low antibody titre was initially observed on Day 5 p.i and a higher titre on Day 7 p.i. From this data, it can be deduced that antibody against the leptospiral infection can develop as early as Day 5. It has been reported that seroconversion will not be seen in the early stage of leptospirosis (Levett, 2001). The antibody of the guinea pigs before infection was 0 titre, if the antibody is >4 at

Day 5 and 7, there is seroconversion (a 4-fold increase in titre). In this study it was shown that there was absence of seroconversion against the leptospires in the guinea pigs on Day 1, 2 and 3. This is related to the lag period of antibody production in the host, where antibody especially IgG would only be detectable after the first week following infection (Appassakij *et al.,* 1995). The first week of illness is the leptospiraemic phase, where the pathogen is distributed and replicating in all organs resulting in leptospiraemia (Levett, 2001).

The detection of leptospires in the liver by PCR assay suggested that the liver is a conducive environment for leptospires to localize and multiply (De Brito *et al.,* 1979) and the detection of the pathogens by this technique indicates that the leptospires primarily infect liver, they multiply and then become bacteraemia to infect other organs. This study explains that leptospires were able to multiply and spread to various organs before the antibody titre is elevated and expressed their virulence inside the tissues resulting in lesions and related clinical symptoms.

CONCLUSION

This present study disclosed major clinicopathological changes caused by *Leptospira icterohaemorrhagiae* serovar Lai strain Langkawi on the infected guinea pigs. It is believed that the same changes could happen when pathogenic *Leptospira* infect humans or other animal species.

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