UNIVERSITI PUTRA MALAYSIA

CLINICOPATHOLOGICAL CHANGES IN GUINEA PIGS (Cavia porcellus linnaeus) FOLLOWING INFECTION BY Leptospira icterohaemorrhagiae SEROVAR Lai STRAIN LANGKAWI

TYAGITA

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By

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MASTER OF VETERINARY SCIENCE
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DEDICATION

This thesis is dedicated to my beloved parents, Edhy Hartady and Nina Sutriana; and my brother, Chiko Hartady, for their devotion and encouragement which provided me the strength and resilience throughout the course of this study. I love you all.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master Veterinary Science

CLINICOPATHOLOGICAL CHANGES IN GUINEA PIGS (*Cavia porcellus linnaeus*) FOLLOWING INFECTION BY *Leptospira icterohaemorrhagiae* SEROVAR *Lai* STRAIN LANGKAWI

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Chairman: Prof. Dato'. Abdul Rani Bahaman, PhD

Faculty: Faculty of Veterinary Medicine

Leptospirosis is a zoonosis of worldwide distribution caused by infection with pathogenic *spirochetes* of the genus *Leptospira.* Interaction between human and mammalian reservoir is the main factor of the transmission of the disease, where human can be infected through direct contact with the infected animals or through exposure to fresh water or soil contaminated by infected animal urine. Unfortunately, the number of cases of leptospirosis in humans is apparently under reported. Information on leptospiral infection in domestic animals is still lacking and this could impede the understanding of the epidemiology and pathogenesis of the disease which later allows the implementation of control programs of leptospiral infection in Malaysia. This study was expected to learn the clinical symptoms and pathological changes in guinea pigs infected with

Leptospira icterohaemorrhagiae serovar *Lai* strain Langkawi; and provide a better understanding of the pathogenesis of leptospirosis.

Three-week old of 17 guinea pigs (*Cavia porcellus linnaeus*) of approximately 250-300 grams body weight were used in this study. There were five treated groups (n=3), injected with 10^6 of low-passage *L. icterohemorrhagiae* serovar *Lai* strain Langkawi and two guinea pigs were injected with EMJH liquid intraperitoneally as negative control. The animals were observed and recorded daily for clinical signs (started from the animal arrived to the animal house and during the experiments processed), which include presence of discharge, respiratory distress, body temperature, mucous membrane and icterus. The animals were sacrificed serially beginning from Day 1 until day 7 p.i. The negative control guinea pigs were sacrificed on Day 0 and Day 7 p.i. Blood samples were taken for MAT pre (Day 0) and post infections (Days 1, 2, 3, 5 and 7 p.i) from both groups, while the lung, liver, kidney and spleen were removed from 3 sacrificed guinea pigs per each serial killing day (on Days 1, 2, 3, 5 and 7 p.i) for light microscopy (H&E stain and silver stain), ultrastructural (transmission electron microscopy) and molecular studies (polymerase chain reaction).

Alteration of body temperature, dehydrated, droopy eyes and jaundice, were noted since day 1 until day 7 p.i. Sudden death was found in guinea pig on day 5 and day 7 p.i. Hematology and biochemistry test result were observed low level of total white blood cells (WBC), neutrophils and lymphocyte significantly on day 7 p.i. The decline of thrombocytes, red blood cells (RBC) and hemoglobin (Hb) were also noted, however it was not significant. There were enhancement level of electrolytes such as sodium (Na),

Chlore (Cl), and potassium (K) insignificantly. The data also mentioned that the level of aspartate aminotransferase (AST), both total bilirubin and conjugated bilirubin were increased. For albumin, alanine transaminase (ALT), blood urea nitrogen (BUN), total protein and creatinine showed the opposite result. While significant changes was can be seen in the low alkaline phosphatase (ALP) gradually.

Histopathological changes such as congestion, hemorrhages and edema were overlooked in all represent organs (lung, liver, kidney and spleen) started since day 1 p.i, contained inflammatory cells infiltration (neutrophil, lymphocytes and macrophages), degenerated and necrotic cells, vascular wall injuries and hemolysis. The chronic active hepatitis which occurred on day 5 p.i, had altered blood circulation through fibrosis. Thrombi were found in the liver and glomerular capillaries. Hydropic degeneration indicating progressive ischemia in the livers and kidneys were observed on day 5 up to day 7 p.i.

On silver staining, leptospires were found adjacent to the central veins and in bile canaliculi at sites of centrilobular necrosis in the liver. In the kidneys, leptospires were detected in the interstitium adjacent to renal corpuscles, proximal and distal tubules and also collecting tubules of the kidneys. Leptospires were also detected in the red and white pulp of the spleen, sinusoid of the red pulps, lymphocytes and vascular wall both in red and white pulp.

Transmission electron microscopy (TEM) examination of the lungs showed that *Leptospira* was only observed outside the alveolar capillary on Day 3 p.i. Necrotic white pulps were evidenced by cellular degeneration on Day 7 p.i as a result under the electron

microscope, no intracellular *Leptospira* was seen in the hepatocytes. However, *Leptospira* were observed adjacent and appeared attached to the hepatocyte cell membrane on Day 3 p.i and the *Leptospira* captured in sinusoid causing ruptured membrane of the hepatocytes on Day 5 p.i. In the kidneys, *Leptospira* was adjacent to degenerated tubular cells at the interstitial junction.

Serum samples from the guinea pigs (negative control and the treated groups) were subjected to the microscopic agglutination test (MAT). Antibody against the *Leptospira* could be seen in serum samples of the guinea pigs sacrificed on Day 5 p.i (2 guinea pigs) and on Day 7 p.i (2 guinea pigs). The lowest seropositivity was 1:40 for guinea pigs sacrificed on Day 5 p.i, while the highest one was 1:320 for guinea pigs that were sacrificed on Day 7 p.i. The PCR findings revealed *Leptospira* were in the liver on Day 3 p.i and in the kidney on day 7 p.i. All findings were suggestive of leptospirosis which fulfills the objectives of this study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Perubatan Veterinar

PERUBAHAN PATOLOGI-KLINIKAL TERHADAP PENULARAN *Leptospira icterohaemorrhagiae* SEROVAR *Lai* STRAIN LANGKAWI DI DALAM GUINEA PIG *(Cavia porcellus linnaeus)*

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Leptospirosis adalah zoonosis yang tersebar di seluruh dunia yang disebabkan oleh jangkitan patogenik *spirochaetes* daripada genus *Leptospira*. Interaksi antara reservoir manusia dan mamalia adalah faktor utama transmisi penyakit ini, dimana manusia boleh dijangkiti melalui sentuhan langsung dengan hewan yang dijangkiti atau melalui pendedahan kepada air tawar atau tanah yang tercemar oleh air kencing haiwan yang dijangkiti. Malangnya, bilangan kes leptospirosis pada manusia nampaknya kurang dilaporkan. Maklumat mengenai jangkitan leptospiral pada haiwan domestik masih kurang dan ini boleh menghalang pemahaman epidemiologi dan patogenesis penyakit yang kemudiannya membolehkan pelaksanaan program kawalan jangkitan leptospiral di Malaysia. Kajian ini telah dijangka untuk mempelajari tanda-tanda klinikal dan perubahan patologi pada tikus belanda yang dijangkiti *Leptospira icterohaemorrhagiae*

serovar *Lai* strain Langkawi; dan memberi kefahaman yang lebih mengenai patogenesis leptospirosis.

Tikus belanda *(Cavia porcellus linnaeus)* sebanyak 17 ekor, usia 3 minggu dengan berat badan berkisar 250-300 gram, telah digunakan dalam kajian ini. Terdapat 5 kumpulan yang di beri perlakuan (n=3), disuntik dengan 10^6 laluan-rendah *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi. Dua tikus belanda lainnya dalam kumpulan kontrol negatif telah disuntik intraperitoneal dengan 500 µl EMJH cair. Haiwan diperhatikan dan direkodkan setiap hari dengan untuk tanda-tanda klinikal seperti kehadiran discharge, masaalah pernapasan, perubahan suhu badan, mucus membran dan icterus. Haiwan dikorbankan bersiri bermula dari Hari ke 1 sampai dengan ke 7 p.i. Kawalan tikus belanda daripada kumpulan kontrol negatif dikorbankan pada Hari ke 0 dan 7 p.i (1 ekor tiap hari pengorbanan). Sampel darah di ambil untuk MAT pada waktu sebelum (Hari ke 0) dan sesudah jangkitan (Hari ke 1, 2, 3, 5 dan 7 p.i) daripada kedua grup; manakala paru-paru, hati, buah pinggang dan limpa dikeluarkan daripada 3 tikus belanda yang diinfeksi pada tiap-tiap hari pengorbanan untuk pemeriksaan menggunakan mikroskop cahaya (dengan pewarnaan H&E dan pewarnaan silver), ultrastruktural (transmission electron microscopy), dan kajian molekul (polymerase chain reaction).

Perubahan suhu badan, dehidrasi, mata sayu dan jaundis diperhatikan sejak Hari ke 1 sehingga Hari ke 7 p.i. Dua ekor tikus belanda ditemui mati mengejut pada Hari ke 5 dan 7 p.i. Hasil daripada ujian hematologi dan biokimia diperoleh level total sel darah putih, neutrophil and lymphocytes adalah rendah yang secara signifikan dapat dilihat pada Hari ke 7 p.i. Penurunan trombosit, sel darah merah (RBC), dan hemoglobin (Hb)

juga didapati, namun ia tidak begitu ketara. Terdapat peningkatan level elektrolit dalam darah seperti natrium (Na), klorine (Cl) dan kalium (K) secara tidak ketara. Data juga menyebutkan bahawa level aspartate aminotransferase (AST), total bilirubin dan conjugated bilirubin meningkat. Bagi albumin, alanine transaminase (ALT), urea nitrogen dalam darah (BUN), total protein dan creatinine menunjukkan hasil yang bertentangan. Manakala perubahan besar boleh dilihat pada level alkaline phosphatase (ALP) yang menurun secara berangsur-angsur.

Perubahan histopatologi seperti kongesti, hemorrhage dan edema tampak dalam organ perwakilan yang diperiksa, terdapat penyusupan sel-sel radang (netrofil, limfosit dan makrofaj), sel rosak dan mati, kecederaan dinding vaskular dan hemolysis. Hepatitis kronik aktif yang berlaku pada Hari ke 5 p.i, telah merubah aliran darah melalui fibrosis. Thrombi ditemui di dalam hati dan kapilari glomerulus. Degenerasi hidropik menunjukkan progresif iskemia dalam hati dan buah pinggang yang diperhatikan pada Hari ke 5 sampai 7 p.i.

Pada pewarnaan perak, sejumlah *Leptospira* didapati bersebelahan dengan central vein dan dalam kanalikuli hempedu di tapak nekrosis centrilobular di dalam hati. Di dalam buah pinggang, *Leptospira* dikesan dalam interstitium, bersebelahan dengan sel renal, tubul proksimal dan distal dan collecting ducts daripada buah pinggang. *Leptospira* juga dikesan dalam pulpa merah dan putih daripada limpa, sinusoid daripada pulpa merah, limfosit dan dinding pembuluh darah di kedua-dua pulpa merah atau putih.

Pemeriksaan paru-paru dengan TEM menunjukkan bahawa *Leptospira* hanya ditemui di luar alveolar kapilar pada Hari ke 3 p.i. Nekrotik pulpa putih yang telah dibuktikan dengan adanya degenerasi sel pada Hari ke 7 p.i, diamati dengan menggunakan TEM, tiada *Leptospira* intraseluler yang dijumpai dalam hepatosit. Walau bagaimanapun, *Leptospira* dijumpai bersebelahan dan muncul berlampiran kepada sel membrane hepatosit pada Hari ke 3 p.i dan *Leptospira* yang ditangkap di sinusoid dengan menyebabkan pecahnya sel membran daripada hepatosit padah Hari ke 3 p.i dan *Leptospira* yang ditangkap di sinusoid dengan menyebabkan mana-mana membran hepatosit pecah pada Hari ke 5 p.i. Dalam buah pinggang, *Leptospira* dijumpai bersebelahan dengan sel-sel tubular yang mengalami degenerasi di interstitial junction.

Sampel serum daripada tikus belanda tertakluk pada ujian microagglutination test (MAT). Antibodi terhadap *Leptospira* boleh dilihat dari sampel-sampel serum tikus belanda yang dikorbankan pada Hari ke 5 (2 ekor) dan Hari ke 7 p.i (2 ekor). Seropositiviti yang terendah ialah 1:40, bagi tikus belanda yang dikorbankan pada Hari ke 5 p.i, dan yang tertinggi ialah 1:320, bagi tikus belanda yang dikorbankan pada Hari ke 7 p.i. Temuan polymerase chain reaction (PCR) mendedahkan *Leptospira* di dalam hati pada Hari ke 3 p.i dan di dalam buah pinggang pada Hari ke 7 p.i. Semua temuan jangkitan leptospirosis memenuhi objektif kajian ini.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of **Master of Veterinary Science.** The members of the Supervisory Committee were as follow:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or any other institutions.

TYAGITA

Date: 5 March 2012

TABLE OF CONTENTS

		Page
ABSTRACT		iii
ABSTRAK		vii
ACKNOWLEDGEMENTS		xiii
APPROVAL		xiii
DECLARATION		xv
LIST OF TABLES		viii
LIST OF FIGURES		xix
LIST OF ABBREVIATIONS		xxix
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	5
	2.1 Structure of *Leptospira spp.*	5
	2.2 Leptospirosis in Human	7
	2.3 Leptospirosis in Animals	8
	2.4 Leptospirosis as a Zoonosis Disease	10
	2.5 Natural Leptospiral Infection	10
	2.6 Experimental Leptospiral Infection	12
	2.7 Clinical Signs	13
	2.8 Pathology of Leptospirosis	15
	2.8.1 Histopathology	15
	2.8.2 Ultrastructural Changes	16
	2.9 Pathogenesis of Leptospirosis	17
	2.10 Immunity	20
	2.11. Diagnosis	20
	2.11.1 Isolation and Identification	21
	2.11.2 Serological Diagnosis	21
	2.11.3 Molecular Methods	22
3	**MATERIALS AND METHODS**	
	3.1 *Leptospira* Strain	25
	3.2 Animals	26
	3.3. Clinical Observation	26
	3.4. Sample Collection and Postmortem	27
	3.5. Hematology and Clinical Biochemistry	27
	3.5.1. Statistical analysis	28
	3.6. Histopathology	28

3.6.1. Light Microscopy Examination 28
3.6.2. Transmission Electron Microscopy (TEM) Examination 29
3.7. Microscopic Agglutination Test (MAT) 30
3.8. Polymerase Chain Reaction (PCR) 31
3.8.1. DNA Extraction 31
3.8.2. Primer Design 32
3.8.3. Agarose Gel Electrophoresis System 32

4 **RESULTS**
4.1. Clinical Observation 34
4.2. Hematology and Clinical Biochemistry 39
4.3. Gross and Histopathological Findings 43
4.3.1. Lungs 43
4.3.2. Livers 53
4.3.3. Kidneys 67
4.3.4. Spleens 79
4.4. Microscopic Agglutination Test (MAT) 91
4.5. Polymerase Chain Reaction (PCR) 92

5 **DISCUSSION** 94

6 **SUMMARY, GENERAL CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH** 106

REFERENCES/BIBLIOGRAPHY 107
APPENDICES 123
BIODATA OF STUDENT 134
LIST OF PUBLICATIONS 135

LIST OF TABLES

Table		Page
4.1	Clinical Signs of Guinea Pigs Post Infection with *L.icterohemorrhagiae* serovar *Lai* strain Langkawi	35
4.2	Haematological values of Guinea Pigs Following Infection with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi	40
4.3	Blood Biochemistry of Guinea Pigs Following Infection with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi	42
4.4	MAT results	91

LIST OF FIGURES

Figure		Page
4.1	Photograph of an infected guinea pig on Day 5 p.i, showing yellow discoloration of the subcutaneous tissue.	43
4.2	Photograph of the lung of a guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 1 p.i, showing mild reddening of the lung (mild pulmonary congestion).	44
4.3	Photomicrograph of a lung tissue of a guinea pig on Day 1 p.i. Note the interalveolar septa are thickened with dilated and congested alveolar capillaries. There is proliferation of pneumocytes type II and scanty infiltration with lymphocytes. H and E. 1,000x magnification.	44
4.4	Photograph of the lung of a guinea pig sacrificed on Day 2 p.i, showing a moderate congestion.	46
4.5	Photomicrograph of a lung tissue of a guinea pig on Day 2 p.i, showing severely thickened interalveolar septa and several lveolar congestion. The thickened alveolar septae cause smaller alveolar lumina. H and E. 100x magnification.	46
4.6	Photomicrograph of a lung tissue of guinea pig on Day 2 p.i, showing alveolitis and alveolar congestion. There are scanty infiltration of the alveolar walls with neutrophils and lymphocytes. Alveolar macrophages are quite prominent. H and E. 1,000x magnification.	47
4.7	Photomicrograph of a lung tissue of guinea pig on Day 3 p.i. Note the blood vessel wall at the tunica media is thickened. Nutrophils are seen marginating the wall of this blood vessel. Early thrombi formation is seen as homogenously reddened areas which are attached to the wall of the vessel. H and E. 1,000x magnification.	47
4.8	Electron micrograph of a lung tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i, showing *Leptospira* inside the alveolar capillary. Lead citrate and uranyl acetate stain, TEM 3,300x magnification.	48
4.9	Lower magnification of a lung tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i,	48

showing neutrophils adjacent to a *Leptospira*. Lead citrate and uranyl acetate stain, TEM 1,700x magnification.

4.10 Photograph of the lung of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i. Note multifocal areas (lobular pattern) of hemorrhages of the apical, cardiac and diaphragmatic lobes. 49

4.11 Photomicrograph of a lung tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing a necrotic bronchiole with detachment of necrotic cells epithelium into the bronchiolar lumen. Perivascular fibrosis and thickening of the necrotic blood vessel wall is also seen. There is also proliferation of tunica media cells which results in smaller lumen of the affected blood vessel. H and E. 200x magnification. 50

4.12 Photomicrograph of a lung section of a guinea pig sacrificed on Day 5 p.i. Rupture of alveolar septae indicating emphysema is seen. Interstitial pneumonia with infiltration of neutrophils at the interalveolar septa was evident. H and E. 200x magnification. 50

4.13 Photograph of the lung of a guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i. Note that the areas of hemorrhages are more extensive, involving all lung lobes. 51

4.14 Photomicrograph of a lung section of a guinea pig sacrificed on Day 7 p.i. Severe focal hemorrhage into the alveolar lumina and emphysema are evident. H and E. 100x magnification. 52

4.15 Photomicrograph of a lung section of a guinea pig sacrificed on Day 7 p.i showing a bronchiole epithelial hyperplasia. Note perivascular fibrin deposition of adjacent blood vessel. H and E. 400x magnification. 52

4.16 Photomicrograph of a lung tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i. Neutrophils have infiltrated the alveolar wall (alveolitis). Oedema fluid with fibrin is observed in the alveolar lumina. H and E. 1,000x magnification. 53

4.17 Photomicrograph of a liver section of a guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 1 p.i. Note thickening of vascular wall. There is also necrosis of vascular layers and perivascular fibrosis. H and E. 400x magnification. 54

4.18	Photomicrograph of a liver section of a guinea pig sacrificed on Day 1 p.i. showing hydropic degeneration and karyolysis of hepatocytes. Kupffer cells are swollen. H and E. 1,000x magnification.	54
4.19	Photomicrograph of a liver section of a guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 1 p.i. Note central vein congestion, severe degeneration and necrosis of hepatocytes. The severely swollen hepatocytes (hydropic degeneration) led to loss of sinusoidal architecture. H and E. 400x magnification.	55
4.20	Photomicrograph of an artery of a liver tissue of a guinea pig sacrificed on Day 1 p.i. The arteriolar wall is thickened and necrotic and there is proliferation of intimal cells with hyperchromatic elongated nuclei which projects into the reduced vascular lumen size. H and E. 1,000x magnification.	55
4.21	Photograph of the liver of guinea pig sacrificed on Day 2 p.i showing a yellowish brown liver.	56
4.22	Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i showing severe degeneration and pyknosis of hepatocytes and swollen kupffer cells. H and E. 400x magnification.	57
4.23	Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i. Note severely thickened arteriolar wall. Many hepatocytes near the thickened artery are necrotic leaving a space around the portal triad replaced by fibrous tissue (periportal fibrosis). The periportal area at the triad has total loss of sinusoidal architecture. H and E. 200x magnification.	57
4.24	Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i, showing leptospires between hepatocytes in the bile canaliculi, adjacent to a central vein. Some leptospires are seen close to the lumen of the central vein. Silver stain. 400x magnification.	59
4.25	Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i, showing hepatic congestion. Many red blood cells are seen in the central vein. H and E. 100x magnification.	59
4.26	Photomicrograph of a liver tissue of guinea pig infected with *L.*	60

icterohaemorrhagiae serovar *Lai* strain Langkawi on Day 3 p.i. Note loss of continuity of the central vein. Hepatocytes around the central vein are atrophied and necrotic. A few neutrophils are seen present at site where hepatocytes are necrotic. H and E. 400x magnification.

4.27 Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i, showing necrotic (karyolysis) bile duct, thickening, degeneration evidence of spaces) and necrosis of arteriolar wall and intimal proliferation. There are also perivascular haemorrhage and fibrosis. H and E. 400x magnification. 60

4.28 Electron micrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i, showing leptospires close to hepatocyte. The leptospires are seen closely attached to the cell membrane. At sites of adherence, the cell membrane appeared to be invaginated. Lead citrate and uranyl acetate stain. TEM, 2,400x magnification. 61

4.29 Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing vasculitis of the vein (phbletis) and periportal fibrosis and necrosis. Spaces of the inflamed vessel wall were also evident indicating degenerative changes. H and E. 100x magnification. 62

4.30 A higher magnification of Figure 4.29. A liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i. Note hyperplasia of the bile duct epithelial cells. Many of these cells appeared to detach into the lumen of the bile duct. A thrombus is seen very closely attached to the wall of this artery. H and E. 400x magnification. 63

4.31 Photomicrograph of liver a tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i. Note a thickened artery at the portal triad area with necrotic vascular wall and intimal proliferation that slightly obliterated the vascular lumen. H and E. 1,000x magnification. 63

4.32 Electronmicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i. *Leptospira* is seen in the sinusoid. Lead citrate and uranyl acetate. TEM 1,000x magnification. 64

4.33 Photograph of the liver of guinea pig died on Day 7 p.i, showing dark red and yellowish liver. 65

4.34 Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing thickened and necrotic vascular wall. The sinusoid is filled with red blood cells in this congested liver. H and E. 200x magnification. 65

4.35 Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing severe necrosis of blood vessel marked by disappearance of nuclei (karyolysis) of the tunica intima cells. H and E. 400x magnification. 66

4.36 Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing many necrotic hepatocytes near the central vein. Many hepatocytes are also seen with hydropic degeneration. H and E. 200x magnification. 66

4.37 Photomicrograph of a liver tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing many *Leptospira* in the bile canaliculi near the central vein. One end of a *Leptospira* is in the lumen of the central vein. Silver stain. 1,000x magnification. 67

4.38 Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day1 p.i, showing a very congested kidney and hemorrhage at the interstitial area near convoluted tubules and renal corpuscle. H and E. 200x magnification. 68

4.39 Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 1 p.i, showing hemorrhage at the interstitium and vacuolation of the renal tubule at higher magnification. H and E.400x magnification. 68

4.40 Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i, showing renal congestion and hemorrhage. H and E. 100x magnification. 69

4.41 Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i, showing karyolysis and hydropic degeneration of tubular cells. H and E. 400x magnification. 70

4.42	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i showing necrotic glomerulus marked by the necrosis of mesangial cell. The Bowman's capsule is ruptured evidence with hemorrhage. H and E. 1,000x magnification.	70
4.43	Photomicrograph of kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i, showing several *Leptospira* at the interstitium of the collecting tubule. Silver stain. 400x magnification.	71
4.44	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i. Note degeneration (swollen cells) of renal tubular cell causing decreased renal tubular lumina. The red blood cells can be seen in the interstitium (hemorrhage). H and E. 200x magnification.	72
4.45	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i, showing atrophied and necrotic glomerulus. H and E. 400x magnification.	72
4.46	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i. Note many leptospires at the interstitial areas in between collecting tubules. Silver stain. 400x magnification.	73
4.47	Photograph of a kidney of guinea pig infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing enlarged kidney with yellow discoloration of renal pelvis fat. The border between cortex and medulla a	74
4.48	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i. Note focal mononuclear infiltration (interstitial nephritis). H and E. 200x magnification.	74
4.49	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i at higher magnification of Figure 4.49, showing lymphocytes and macrophage infiltration. H and E. 1,000x magnification.	75
4.50	Photomicrograph of kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing leptospires in the interstitium between swollen tubules. Silver stain. 400x magnification.	75

4.51	Electronmicrograph of kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i. Note *Leptospira* at the intercellular junction between renal tubular cells. Adjacent tubular cell shows distention of the rough endoplasmic reticulum. Mitochondria appeared to loose their normal cristae arrangement. The tubular lumen contains cellular debris. Lead citrate and uranyl acetate. TEM 4,000x magnification.	76
4.52	Photograph of kidney of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7p.i, showing pale-yellowish renal pelvis. The border between the cortex and medulla is indistinct and moderately congested.	77
4.53	Photograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing pyknotic and karyolytic tubular cells. The tubular cells contain spaces seen in hydropic degeneration which results in the cells to become swollen and obliterate the tubular lumen. H and E. 400x magnification.	77
4.54	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i. Note focal inflammatory cells infiltration (chronic interstitial nephritis) and perivascular fibrosis. H and E. 100x magnification.	78
4.55	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing higher magnification of focal inflammation with lymphocyte infiltration and perivascular fibrosis. The blood vessel wall is thickened, necrotic (pyknosis and karyolysis) and showed presence of spaces (degeneration). H and E. 200x magnification.	78
4.56	Photomicrograph of a kidney tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing leptospires at the interstitium around the collecting tubules. Silver stain. 400x magnification.	79
4.57	Photograph of a spleen of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 1 p.i, showing mild congestion of the spleen.	80
4.58	Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 1 p.i, showing that blood vessels and the venous sinuses are packed with red blood cells (congested areas). H and E. 100x magnification.	80

4.59	Photograph of a spleen of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i, showing moderate congestion of the spleen.	81
4.60	Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i, showing splenic congestion in the red pulp. H and E. 100x magnification.	81
4.61	Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 2 p.i, showing higher magnification of Figure 4.61. There are infiltration by macrophages, neutrophils and lymphocytes in the red pulp. Red blood cells were packed in the sinusoid. Red blood cells were packed in the sinusoid. H and E. 1,000x magnification.	82
4.62	Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 3 p.i, showing severe congestion of the red pulp. H and E. 100x magnification.	83
4.63	Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi which sacrificed on day 3 p.i, showing higher magnification of Figure 4.62, showing sinusoids filled with RBC and mild neutrophil infiltration. H and E. 1,000x magnification.	83
4.64 (A-B)	Photographs of spleens of negative control (A), showing a dark red spleen while (B) is a spleen of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing bright red and enlarged spleen.	84
4.65	Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing a necrotic (karyolysis and pyknosis) white pulp (coagulation necrosis) and infiltration of several neutrophils (splenitis) at this foci which is also hemorrhagic. H and E. 400x magnification.	85
4.66	Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing interstitial oedema and focal necrotic area. Neutrophils and macrophage is seen present in the white pulp. H and E. 1,000x magnification.	85
4.67	Photomicrograph of a spleen tissue of guinea pig infected with *L.*	86

icterohaemorrhagiae serovar *Lai* strain Langkawi on Day 5 p.i, showing leptospires in the central artery and interstitium of the white pulp. Silver stain. 400x magnification.

4.68 Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, showing higher magnification of Figure 4.69. The leptospires were attached into lymphoid cells and blood vessel wall. Silver stain. 1, 000x magnification. 86

4.69 Electronmicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 5 p.i, *Leptospira* adjacent to an endothelial cells with margination of its chromatin. The long arrows are pointing to transverse sections of blood vessels. Lead citrate and uranyl acetate. TEM 1,700x magnification. 87

4.70 Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing necrotic foci which is hemorrhagic in the white pulp. H and E. 100x magnification. 88

4.71 Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing neutrophils and lymphocytes infiltration in necrotic red pulp. H and E. 1,000x magnification. 88

4.72 Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing leptospires in the red pulp. Silver stain. 400x magnification. 89

4.73 Photomicrograph of a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing higher magnification of *Leptospira* attaches intercellular in red pulp. Silver stain. 1,000x magnifications. 89

4.74 Electronmicrograph a spleen tissue of guinea pig infected with *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi on Day 7 p.i, showing necrosis of a lymphocyte. The cell membrane ruptures, the cristae of the mitochondria are disorganized and the rER disintegrated. Lead citrate and uranyl acetate. TEM 2,100x magnification. 90

4.75 PCR results of liver and kidney samples of the guinea pigs experimentally infected with *L. icterohaemorrhagiae* serovar *Lai* 93

strain Langkawi.

LIST OF ABBREVIATIONS

%	Percentage
&	And
bp	Base pairs
ACUC	Animal care and use committee
AE	Elution buffer
AL	Cell lysis buffer
Alb	Albumin
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate aminotransferase
ATL	Animal tissue lysis
AW	Column wash buffer
B. Neut	Band neutrophil
BSA	Bovine serum albumin
BT	Body temperature
^{o}C	Degrees Celsius
CK	Conjugated creatinine
Cl	Chlorine
ELISA	Enzyme-linked immunosorbent assay
D. Bil	Direct bilirubin
DNA	Deoxyribonucleic acid
DPX	Di-N-butyle phthalate in xylene

EDTA	Ethylen ediamine tetraacetic acid
EMJH	Ellinghausen and McCullough Johnson media
GA	General appearance
Hb	Hemoglobin
HCl	Hydrochloric acid
H&E	Hematoxylene eosin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMR	Institute for medical research
K	Kalium (potassium)
Lymp	Lymphocyte
LPS	Lipopolysaccharide
LSD	Least significant difference
µ	Microliter
µm	Micrometer
MAT	Microscopic agglutination test
ml	Milliliter
mAbs	Monoclonal antibodies
M	Molar
MM	Mucosa membrane
Na	Natrium (sodium)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume

pH	Puissance hydrogen (hydrogen ion concentration)
p.i	Post infection
RBC	Red blood cell
rER	Rough endoplasmic reticulum
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic acid
SD	Standard deviation
S. Neut	Segmented neutrophil
SS	Silver stain
ST	Skin turgor
TAE	Tris – Acetate – EDTA
T. Bil	Total bilirubin
TEM	Transmission electron microscopy
TP	Total protein
WBC	White blood cell
V	Voltage

CHAPTER 1

INTRODUCTION

Leptospirosis is a zoonotic disease of worldwide distribution (Trevejo *et al.*, 1998), caused by pathogenic *spirochetes* of the genus *Leptospira* which has a large number of species. These species have been regrouped into two entities: *Leptospira interrogans* sensu lato, which includes pathogenic species and *Leptospira biflexa* sensu lato, comprising non pathogenic species and freshwater *saprophytes*. More than 200 serotypes of *L. interrogans* have been identified. In Malaysia, thirty-seven leptospiral serovars from thirteen serogroups have been bacteriologically identified initially. The thirteen serogroups are: *Australis, Autumnalis, Bataviae, Canicola, Celledoni, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Sejroe* and *Tarassovi* (Bahaman and Ibrahim, 1988). However in 2006, the Spirochete Group in Pasteur Institute, France, has classified serovars of *Leptospira* identified in Malaysia as: *Fugis, Mooris, Jonsis, Gurungi, Muelleri, Burkini, Smithi, Abramis, Biggis, Evansi, Hemolytica* and *Ricardi* (Spirochete Group at Pasteur University, 2006).

Interaction between mammalian reservoirs and human is the main factor in the transmission of the disease, where humans can be infected through direct contact with infected animals or through exposure to fresh water or soil contaminated by urine of infected animals (Orpilla-Bautista and Panaligan, 2002).

The clinical manifestations of leptospirosis ranged from mild febrile illness to icteric-hemorrhages and may be accompanied by severe involvement of filter organs such as the liver, lungs, and kidneys (Plank and Dean, 2000). However, in one study, jaundice and hemorrhage were not commonly manifested (Kobayashi, 2001). With an incubation period between 5 to 14 days (Orpilla-Bautista and Panaligan, 2002), leptospirosis often goes unrecognized because of its non-specific presentation and had often been misdiagnosed as dengue or malaria (Wuthiekanun *et al.,* 2007).

It is been known that the basic pathomorphological changes in leptospirosis are endothelial damages, which leads to generalized vasculitis (Tappero *et al.,* 2000). *Leptospira icterohaemorrhagiae* can migrate through the kidneys of the rat. During the first four days, the organisms migrate from the capillary lumina to the interstitial tissues and cause interstitial oedema. By the 10th day, the organism can be seen between the epithelial cells of the proximal convoluted tubules and by the 14th day, many are located within the tubular lumina. There is no evidence of viable *Leptospira* within the cells of the proximal tubules, though occasionally structures resembling leptospiral fragments inside lysosomes are observed (Marshall, 1974).

The use of an animal model to study leptospirosis requires a suitable susceptible animal such as guinea-pigs (*Cavia porcellus linnaeus*) and hamsters (*Cricetus aureus, Cricetus sinensis*) to identify the strains of *Leptospira* (Levett, 2001). These animals have been used for the biological measurement of virulence or immunity which requires a standardized animal, tissue or cell culture system susceptible to the strains of *Leptospira* to be tested (Faine *et al*., 1999).

Genetic studies have demonstrated that serologically diverse serotypes may be present in the same genetic group. At least seven species of pathogenic leptospires have been identified by nucleotide analysis (Johnson, 1976). Laboratory tests can be conducted to achieve accurate diagnosis and surveillance of leptospirosis. There are two main test categories according to Vijayachari and Sehgal (2006): a) Direct evidences which include organism isolation, expression of *Leptospira*, and amplification of specific fragment of leptospiral DNA and b) Indirect evidences with detection of the antibodies to leptospires. Other categories are bacteriological, microscopic, immunological/ serological and molecular techniques.

Leptospirosis has been recognized as an emerging infectious disease because of the recent large outbreaks associated with recreational activities (Morgan *et al*., 2002; Sejvar *et al.,* 2003). Unfortunately, the number of cases of leptospirosis in humans is apparently under reported and this could be due to an oversight by the medical personnel or due to lack of diagnostic facilities. Information on leptospiral infection in domestic animals is still sketchy and this could impede the understanding of the epidemiology and pathogenesis of the disease which could hinder the implementation of effective control programs for leptospiral infection in Malaysia (Bahaman and Ibrahim, 1988).

The objectives of the present investigation were:

1. To describe the clinical pathological changes and localization *Leptospira icterohaemorrhagiae* serovar *Lai* strain *Langkawi* in guinea pigs following exposure to experimental infection.

2. To describe the clinicopathological changes following experimental infection of guinea pigs to *Leptospira icterohaemorrhagiae* serovar *Lai* strain *Langkawi*.

It is hypothesized that *L. icterohaemorrhagiae* serovar *Lai* strain Langkawi is pathogenic in guinea pigs and would enable studies on clinical and pathological changes of leptospirosis in this animal model.

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