DETECTION OF LEPTOSPIRAL ANTIGEN IN THE LIVERS, SPLEENS AND LUNGS OF HAMSTERS INFECTED WITH *LEPTOSPIRA INTERROGANS*, SEROVAR PYROGENES BY IMMUNOPEROXIDASE TECHNIQUE

ARNON PUDGERD

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (ANATOMY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2008

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ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my major advisor, Associate Professor Dr. Kajee Pilakasiri for kindness, valuable instructive advice, supervision, patient and encouragement in correcting my thesis.

I deeply thank my co-advisor, Associate Professor Dr. Jantima Roongruangchai, Associate Professor Arraya Sangiampong and COL Duangporn Phulsuksombati for their valuable advice, comments and helpful guidance in this thesis.

My sincere appreciation goes to COL Dr. Chaiyaphruk Pilakasiri for being chair person of my thesis defense as well as his valuable advice, comments and helpful guidance in this thesis.

Sincere thanks go to Major Noppadon Sangjan and personel of the Department of Laboratory Animal, Armed Forces Research Institute of Medical Sciences for provision of experimental animals, materials and their kind supports that make this thesis possible.

My thanks also go to Dr. Amornpun Sereemaspun, Genetic Unit, Department of Anatomy, Faculty of Medicine, Chulalongkorn University for his kindness, experimental material and suggestion.

For Mrs. Naree Choosrichom and Miss Kanittar Srisook Department of Pathology, Faculty of Medicine Siriraj Hospital, their kindness, experimental material and suggestion are greatly appreciation.

Special thank gives to Mr. Kasem Koedpuech for his kindness, chemical preparation, specimen and slide preparation. Additionally, I thank all members of the microtechnique unit, Department of Anatomy, Faculty of Medicine Siriraj Hospital for their help and friendship.

My deep thanks go to Miss Sirinun Chaipunko, my nice and friendly lab partner for her support, encouragement and friendly that make my tough work easier throughout my graduate student life. Additionally, thank my friends in the Department of Anatomy for their kind supports. I am very grateful to the Department of Anatomy, Faculty of Medicine Siriraj Hospital where opportunity and knowledge have been given to me.

Finally, I wish to express my deepest thankfulness and appreciation to my parents for their support and understanding throughout the entire course of this study.

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ABSTRACT

The objective of this study was to detect the leptospiral antigens in the liver, spleen and lung tissue sections of hamsters infected with *Leptospira interrogans* serovar pyrogenes. Three infected hamsters of each group were sacrificed at 1 hour, 6 hours and on day 1, 2, 3, 4, 5 and 6 after infection. The tissue of all sacrificed animals were removed and processed for the immunoperoxidase staining technique. Concerning the liver, this staining technique showed the positive golden brown coloration deposited in the cytoplasm and boundary of hepatocytes nearby the portal area in the early groups of infection, and in the late groups they were also found in the hepatocytes located throughout the area between the portal area and central vein. In the portal triad, hepatic sinusoid and central vein appeared the leptospiral antigens with various staining intensities from mild to intensive coloration, and might have been due to the direction of the blood circulation from the portal area to the central vein. The spleen of all infected groups displayed leptospiral antigens in the capsule, trabeculae, white pulp and red pulp which are related to the reported histopathological area. In the infected lung, leptospiral antigens were found in the cytoplasm of the epithelia lining the bronchus, bronchiole and alveolus. The organs studied might suffer from the leptospires and/or their antigens in the blood circulation because the components of blood vessels and red blood cells showed positive stain. Additionally, in the neutrophils appeared leptospiral antigens deposited in the cytoplasm in all infected experimental groups. These cells might have engulfed the leptospires and/or their antigens. The leptospiral antigens were always located in the organs that histopathology indicated were diseased. Therefore, the leptospiral antigens that were found should be a primary cause of the histopathology of all infected organs.

KEY WORDS: LEPTOSPIROSIS / *LEPTOSPIRA INTERROGANS* /
IMMUNOPEROXIDASE STAINING

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การตรวจหาแอนติเจนของเชื้อเลปโตสไปรา ในตับ ม้าม และปอดของแฮมสเตอร์ที่ติดเชื้อ *Leptospira interrogans* ซีโรวาร์ pyrogenes ด้วยเทคนิคอิมมูโนเพอร์ออกซิเดส
(DETECTION OF LEPTOSPIRAL ANTIGEN IN THE LIVERS, SPLEENS AND LUNGS OF HAMSTERS INFECTED WITH *LEPTOSPIRA INTERROGANS*, SEROVAR PYROGENES BY IMMUNOPEROXIDASE TECHNIQUE)

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บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาหาตำแหน่งแอนติเจนของเชื้อเลปโตสไปรา ในเนื้อเยื่อตับ ม้าม และปอดของแฮมสเตอร์ที่ติดเชื้อ *L. interrogans* ซีโรวาร์ pyrogenes กลุ่มละ 3 ตัวที่ถูกฆ่าในชั่วโมงที่ 1 และ 6 และในทุกวันจากวันที่ 1 ถึงวันที่ 6 หลังจากติดเชื้อ โดยนำเอาอวัยวะดังกล่าวที่ติดเชื้อผ่านกระบวนการเพื่อศึกษาตำแหน่งแอนติเจน โดยใช้เทคนิคอิมมูโนเพอร์ออกซิเดส ผลของการศึกษาพบสีน้ำตาลทอง ซึ่งเป็นตำแหน่งของแอนติเจนของเชื้อเลปโตสไปรา ในตับพบแอนติเจนภายใน cytoplasm และรอบนอกของ hepatocyte ที่อยู่ใกล้ portal area ในระยะแรกๆของการติดเชื้อ ส่วนในระยะหลังๆก็จะพบใน hepatocyte ที่อยู่ระหว่าง portal area และ central vein นอกจากนี้ยังปรากฏแอนติเจนที่ portal triad hepatic sinusoid และ central vein ซึ่งความเข้มของสีน้ำตาลทองที่พบนั้นแตกต่างกันและเป็นไปตามทิศทางของกระแสโลหิต ในม้ามจะพบแอนติเจนของเชื้อเลปโตสไปราใน capsule และ trabeculae รวมทั้งเซลล์ใน white pulp และ red pulp ซึ่งสัมพันธ์กับบริเวณที่เกิดพยาธิสภาพ ส่วนในปอดจะพบแอนติเจนของเชื้อเลปโตสไปราใน cytoplasm ของเซลล์ที่บุ bronchus bronchioleและถุงลม ทั้งนี้ม้าม และปอดน่าจะได้รับเชื้อเลปโตสไปราและ/หรือ แอนติเจนจากกระแสโลหิตเช่นเดียวกับตับเพราะหลอดโลหิตและเม็ดเลือดแดงของอวัยวะเหล่านี้ปรากฏแอนติเจน นอกจากนี้ยังพบแอนติเจนภายใน cytoplasm ของเม็ดเลือดขาวชนิด neutrophil ในทุกกลุ่มของการทดลอง ซึ่งเซลล์นี้มาทำหน้าที่กำจัดเชื้อเลปโตสไปรา โดยได้กินแอนติเจนเหล่านี้เข้าไปอยู่ใน cytoplasm โดยสรุปจะพบแอนติเจนของเลปโตสไปราเสมอในบริเวณที่มีรายงานว่ามีพยาธิสภาพเกิดขึ้น ดังนั้นแอนติเจนเหล่านี้น่าจะเป็นสาเหตุหลักของการเกิดพยาธิสภาพในอวัยวะต่างๆที่ติดเชื้อ

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CHAPTER I
INTRODUCTION

History of leptospirosis

Leptospirosis is zoonosis disease worldwide caused by pathogenic spirochetes of the genus *Leptospira* (Dutta *et al*., 2005). The first description of leptospiral infection in human was probably made by Landouzy in 1883 but it was remained for Adolf Weil in 1886 to clearly separate leptospiral jaundice (Arean, 1962). Therefore, this syndrome was reported over 100 years ago (Levett, 2001). The first observation of pathogenic leptospires was described by Stimson in 1907. Stimson studied patients who died with yellow fever by silver staining. They founded microorganism which were named *Spirocheta interrogans* (Dutta *et al*., 2005; Sambasiva *et al*., 2003; Arean, 1962). Leptospirosis was certainly recognized as an occupational hazard of rice harvesting in ancient China (Levett, 2001) which was called "Wei ni" or rice-harvest jaundice while the Japanese name was nanukayami or autumn fever (Faine *et al*., 1999). In Japan, the spirochete was first isolated by Inada *et al*. in 1915 after Adolf Weil had described the clinical disease 30 years (Dutta *et al*., 2005). Therefore, they detected both spirochetes and specific antibodies in the blood of Japanese miners. The importance of occupation as a risk factor was recognized early. The role of the rat as a source of human infection was discovered in 1917. In addition, leptospirosis in livestock was recognized some years later (Levett, 2001). In the 15 years after discovery until the 1930s many of the importance serovars prevalent throughout the world and their host sources were discovered (Sambasiva *et al*., 2003) such as the carriage of serovar bim by house mice (Bharti *et al*., 2003). At present, *Leptospira* spp. is endemic to wild and domestic mammals as well as reptiles and amphibians. Rats and other rodents are the most important sources for human infection (Plank *et al*., 2000). However, the dog plays an important role in the transmission of leptospires to infect human (Venkataraman *et al*., 1992).

Leptospirosis in Thailand was first reported in 1942. Later, in 1972, leptospirosis was included as one of the 58 infectious diseases under the National Passive Surveillance system. The reports of leptospirosis cases were base on the clinical case definition of the World Health Organization (WHO). Ten to twenty of leptospirosis cases were reported annually in Thailand in 1972 to 1988. The number of cases range from 55 to 272 cases per year in between 1982 and 1994 and exhibited an annual incidence rate of approximately 0.3/100,000 population. The case of leptospirosis was reported throughout in 1995 to 2003 and peak in 2000 (Tangkanakul *et al*., 2005). In addition, 2,868 cases were reported during 2005 (Thaipadungpanit *et al*., 2007).

Epidemiology

Leptospirosis has a widespread distribution as well as outbreaks of leptospirosis have been related to heavy rainfall in various part of the world (Plank *et al*., 2000). The incidence of human infection is higher in the tropic region than in temperate region but transmission occurs in both industrialized and developing countries (Levett, 2001; Bharti *et al*., 2003). Two widely endemic zones for leptospirosis exist. The first is the Caribbean and Latin America which the mortality rate was around 10 percent of the patients and may reach 23.6 percent in some countries such as Barbados. The second includes most of the countries and islands of the Indian and the Pacific Ocean (Pappas *et al*., 2006). In 1983, human leptospirosis was first described in New Caledonia (South Pacific). In 1989, leptospirosis incident rate was 90 per 100,000 persons a year with specific mortality rate of 4 percent of patients and the incidence of the rural area was 112 per 100,000 persons a year which was seven times higher than in urban subside. Two periods with higher incidence were noticed corresponding to highest rainfall (Perrocheau *et al*., 1997). In Europe, the disease is rare. Nevertheless, most reports were from Mediterranean countries and Eastern European (Michel *et al*., 2002; Pappas *et al*., 2006). In addition, in the United States, the incidence of leptospirosis was reported about 100-200 cases per year with most occurring outside the continent United States in Hawaii. Leptospirosis had also been reported in California in 1922. In 1982 to 2001, 61 cases were reported to the California Department of Health Services which was averages 2.8 cases per year. In the period of 1997-2001, 34 cases of leptospirosis were reported as the average of 6.8 cases per

year; as a result, the overall incidence of leptospirosis in California appeared to be on the rise (Meites *et al*., 2004). In Israel, leptospirosis had been considered endemic since 1950 with the peak of 3.6 per 100,000 populations in 1960s. During 1970 to 1979, 251 cases were diagnosed with report attack rate of 0.2 per 100,000 populations mainly in agricultural area and 59 cases in 1985 to 1999. The dominant serovars were *L. icterohaemorrhagica* (17 cases), *L. hardjo* (12 cases) and *L. ballum* (12 cases). In 28 cases the diseases were related to occupational mostly in the farm including pig farmers and dairy workers (Kariv *et al*., 2001). In Japan, until 1960, more than 200 peoples were dead from leptospirosis. After 1960, the number of the reported case of leptospirosis patients had rapidly decreased (Yanagihara *et al*., 2007).

However, Leptospirosis has been reported in Southeast Asia (Myint *et al*., 2007). In the Philippines, leptospirosis patients tended to be frequently found in flood-prone areas of urban setting such as Metro Manila. The morbidity in the rural area (Cabatuan, Iloilo) was 147 cases per 100,000 populations. From 1998 to 2001, about 70 percent of 1200 patients were suspected leptospirosis (Yanagihara *et al*., 2007). In Viet Nam, leptospirosis was first report in 1930 (Van *et al*., 1998).

Thailand is the endemic area of the leptospirosis disease especially in the northeast of the country where most of the people are farmers (Kusum *et al*., 2005). The number of leptospirosis cases reported from 1982 to 1995 ranged from 55 to 272 cases per year, with an average incidence of 0.3 per 100,000 per year. The number of human leptospirosis cases reported in 1996 was 398 (incidence of 0.65 per 100,000). In 1997, 2,334 leptospirosis patients (3.83 per 100,000) were observed and 111 patients from 15 provinces in the northeastern region were reported dead. In 1998, the number of cases was 2,230 (3.52 per 100, 000) including 102 deaths, 6,080 (9.89/100,000) in 1999, and 14,286 (23.2 per 100,000) in 2000. Therefore, leptospirosis corresponds with the rainy season, with an increase in cases beginning in August and decreasing in November. The peak number of cases occurs in October (Tangkanakul *et al*., 2000; Phraisuwan *et al*., 2002). In 2001, 10,217 cases were reported including 171 deaths. In 2002, 148 specimens of blood sample from patients that were suspected leptospirosis from the northeast of the country showed 22 specimens (15%) being positive to the culture for leptospires (Kusum *et al*., 2005). From this data, the increase in leptospirosis was reported between 1995 and 2003 with

the peak in 2000. Many serovars of leptospires had been found in human in Thailand. Investigation in the late 1990s indicated the serovar bratislava, sejroe, pyrogenes and autumnalis which had the most serologic reactivity by microscopic agglutination test (MAT) (Myint *et al*., 2007). In addition, the annual incidence of leptospirosis in Takeo province, Cambodia was 7.65 per 100,000 populations in 2003. This incidence was in the same range of leptospirosis in Thailand which was 7.88 per 100,000 populations in 2003. As a result, the Cambodian population had many occupation and environment risk factors similar to Thais. Moreover, Cambodia shares the border with Thailand (North), Viet Nam (East and South) and Laos (Northeast) (Seng *et al*., 2007).

The leptospires are maintained by persistent colonization in the proximal renal tubules of the carrier animal host. An infected animal can remain symptom free and shed infectious organisms in the urine for its lifetime (Bharti *et al*., 2003). Water has been recognized as an importance for transmission of leptospires to human (Gillespie *et al*., 1963). In addition, many animals are reservoir host for several serovars of leptospires (Table 1) (Bharti *et al*., 2003).

The source of infection in human might be direct and indirect contact with the urine of infected animal (Levett, 2001). Leptospires entered the body through broken skin and conjunctiva as well as inhalation of water or aerosols which caused the infection via the mucous membrane of respiratory tract (Levett, 2004). Thus, infection in human might be acquired through occupational and recreational exposure. There was a significant risk associated with direct occupation contact with the infected animals or during recreational water sport (Doudier *et al*., 2006). However, infection from infected animal bite was rare (Levett, 2004). Direct contact with infected animals was found most in farmers, abattoir workers, rodent control workers, meat inspectors and veterinarians. Indirect contact would occur in sewer workers, soldiers, miner, septic tank cleaners, gamekeepers, canal workers, fish farmers, rice field workers, banana farmers, taro farmers and sugar cane cutters (Levett, 2001).

Moreover, leptospirosis could be found in other mammalians including horses, dogs, pigs, cattle (Nally *et al*., 2001), wild animals such as monkeys, camels, platypus and sea lions (Cox *et al*., 2005). In addition, in northeast of Tasmania there was the evidence of leptospirosis in wombats (Munday *et al*., 1973), and flying foxes in Australia (Cox *et al*., 2005).

Table1. Typical reservoir host of common leptospiral serovar (Bharti *et al*., 2003).

Reservoir	Serovars
Pigs	pomona, tarassovi
Cattle	hardjo, pomona
Horses	bratislava
Dogs	canicola
Sheep	hardjo
Racoon	grippotyphosa
Rats	icterrohaemorrhagiae, copenhageni
Mice	ballum, arborea, bim
Marsupials	grippotyphosa
Bats	cynopteri, wolffi

Taxonomy

Leptospires are spirochetes, a group of bacteria that diverged early in bacterial evolution (Bharti *et al*, 2003). They are classified in the order Spirochaetales, the group of flexuous, thin, gram negative, chemoheterotrophic, helical shape organism, which differ morphologically form other prokaryotes by the present of axial fibril (endoflagellum, axistyle or axial filament) (Holt, 1978). Family Leptospiraceae is divided into three genera, *Leptonema*, *Turneria* and *Leptospira* (Baraton *et al*., 1995; Plank *et al*., 2000). The genus *Leptospira* consists of two species, *biflexa* and *interrogans* (Figure 1) (Baraton *et al*., 1995) by using serologic methods. The *L. biflexa* contained saprophytic strains (non-pathogen) and *L. interrogans* comprised all pathogenic strains. The *L. interrogans* is the cause of leptospirosis disease (Levett, 2001). The pathogenic and saprophytic leptospires are similar in morphology but differ in several biological characteristic (Johnson *et al*., 1967) and a number of biochemical tests. Both *L. biflexa* and *L. interrogans* are divided into numerous serovars defined by agglutination after cross reaction with homologous antigen (Table 2) (Levett, 2001; Levett, 2004). The serological classification is still used in laboratory and epidemiological studies (Kositanont *et al*., 2007).

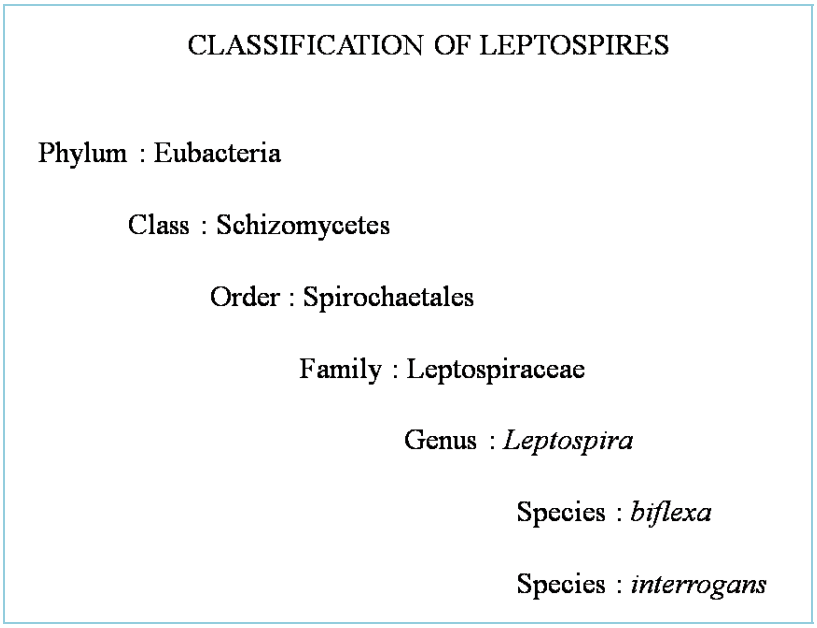


Figure 1. Schematic classification of *Leptospira interrogans* (Base on data reported by Faine *et al*.,1999; Levett, 2001; Bharti *et al*., 2003; Ren *et al*., 2003).

Table 2. Classification of *Leptospira* species (Adapted from Bharti *et al*., 2003)

Species	Serovars
L. interrogans	australis
	bratislava
	bataviae
	canicola
	copenhageni
	hebdomadis
	icterrohaemorrhagiae
	lai
	pomona
	pyrogenes
L. biflexa	patoc

Biology of pathogenic leptospires

The leptospires are aerobic bacteria (Ren *et al*., 2003) and do not multiply outside of the host (McDonough, 2001). The common site of the pathogenic leptospires is the mammalian kidney. The presence of the leptospires in lakes and streams can access to the wildlife and domestic animals that expose to this water (Johnson *et al*., 1967). There survive depends on environmental conditions in which leptospires are found, such as soil and water conditions. They are highly sensitive to drying and to pH changes - pH<6 and pH>8 are inhibitory; temperatures < 7 - 10ºC (44.6 - 50ºF) and > 34 - 36ºC (93 - 96ºF) are detrimental. This organisms survive up to 180 days in wet soil, for many months in surface water and they survive better in stagnant than in free-flowing water (McDonough, 2001). They move by a rapidly drifting rotation, often associated with a flexing or undulating movement along the helical path (Pavia, 1994). Therefore, movement of leptospires may be classified into three types including rotation around the central axis, circular motion and progressive movement in the direction of the straight (Bharti *et al*., 2003). Moreover, the leptospires multiply by transverse binary fission (Czekalowski, 1963). They can produce monounsaturated fatty acids by direct desaturation of corresponding saturated acids but they do not produce polyunsaturated fatty acids by further desaturation (Kondo *et al*., 1972).

Morphology of leptospires

Leptospires are spirochete (Greek: *spira* for "coil" and *chaete* "hair") and have the basic cell wall structure of gram-negative bacteria but they are stained poorly with the gram stain (Rubin *et al*. 1999; Nester *et al*. 2001). The leptospires are tightly coiled with 18 or more coils. They are 0.1 μm in wide and 6 to 12 μm in long (Rubin *et al*., 1999) and have only one axial filament at each end (Wolgemuth *et al*., 2006). The wavelength is proximately 0.5 μm and the helical amplitude is approximately 0.1 – 0.5 μm. They have pointed ends, usually one or both ends of this single cell organism are bent or hooked distinction (Figure 2) (Faine *et al*., 1999; Levett., 2001).

Leptospiral structures

Leptospires have a typical double membrane structure in common with other spirochetes (Figure 3). They are composed of the outer membrane and inner membrane, separated by periplasmic space (Levett, 2001). The electron microscopy demonstrated three basic structures of leptospires which consists of membrane sheath

envelope, protoplasmic cylinder with consist of cell wall and cytoplasmic membrane and axial filament (Figure 4) (Holt, 1978; Miller *et al*., 1962).

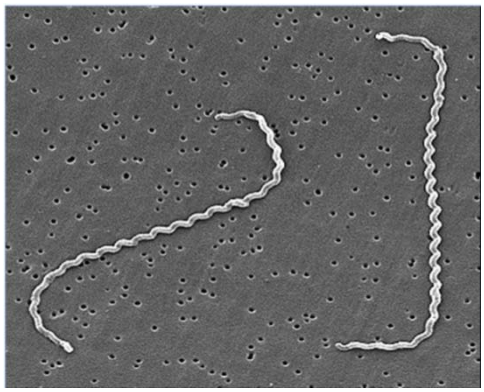


Figure 2. Scanning electron micrograph of *L. interrogans* serovar icterohaemorrhagiae (Levett, 2001).

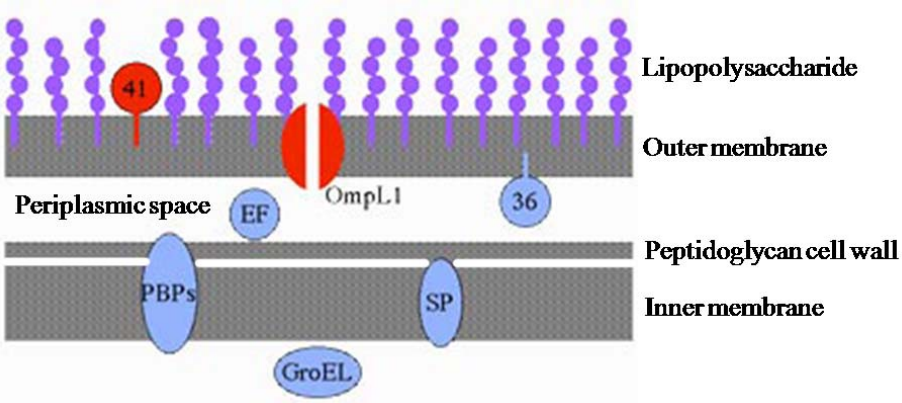


Figure 3. Model of leptospiral membrane architecture shows the location of the outer membrane components, lipopolysaccharide, the porin (OmpL1) and the lipoprotein LipL36 and LipL41. The endoflagella (EF) are located in the periplasmic space, between the outer and inner membranes. The peptidoglycan cell wall is associated with the inner membrane which includes the penicillin-binding protein (PBPs) and signal peptidase (SP). The heat shock protein (GroEl) is located in the cytoplasm (Adapted from Zuerner *et al*., 2000).

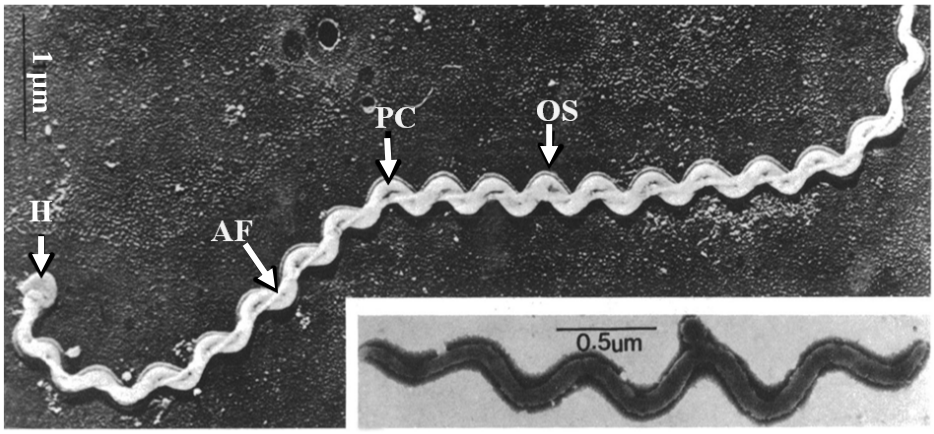


Figure 4. Electron micrograph of *L. interrogans* serovar icterohaemorrhagiae showing hook (H), outer sheath (OS), axial filament (AF), and protoplasmic cylinder (PC) (Adapted from Holt, 1978).

The outer membrane (outer sheath or membrane sheath envelope) envelops the axial filament and the protoplasmic cylinder (Bharti *et al*., 2003). It has approximately 11 nm in wide and has three or five dense light layers demonstrated by electron microscopy (Auran *et al*., 1972). The leptospiral outer membrane is composed of protein, lipid and lipopolysaccharide (LPS) moieties (Faine *et al*., 1999). Therefore, the LPS are the major component of the leptospiral cell surface and are the target antigen for antibodies. The three major proteins in order of relative abundance on the cell surface of leptospires were lipoprotein LipL32, LipL21 and LipL41 which were reported in animal leptospirosis model (Cullen *et al*., 2005).

The chemical analysis of LPS showed the common hexoses, amino hexoses and pentose. Moreover, some sugars were found rarely such as xylose and arabinose. Rhamnose was a sugar component of all serovars (Kondo *et al*., 1972; Zuerner *et al*., 2000) as well as fucose, ribose, glucose, galactose, mannose, galactosamine, glucosamine, mannosamine, glucose-6-phosphate, N-acetyl glucosamine was found (Faine *et al*., 1999). However, the LPS was structurally unique molecules similar to gram-negative bacteria, but have lower toxicity (Levett, 2001; Ren *et al*., 2003). In addition, the leptospiral outer membrane contained few integral transmembrane proteins (Cullen *et al*., 2005)

The axial filament (endoflagella) was organ for locomotor (Faine *et al*., 1999). The axial filaments were enveloped by the outer membrane (Miller *et al*., 1962). In the transverse section of leptospires showed the axial filament located in clear space between the outer membranes and cell wall of the protoplasmic cylinder (Nauman *et al*., 1969; Ritchie *et al*., 1965) which was referred to the periplasm or periplasmic space (Figure 5) (Wolgemuth *et al*., 2006).

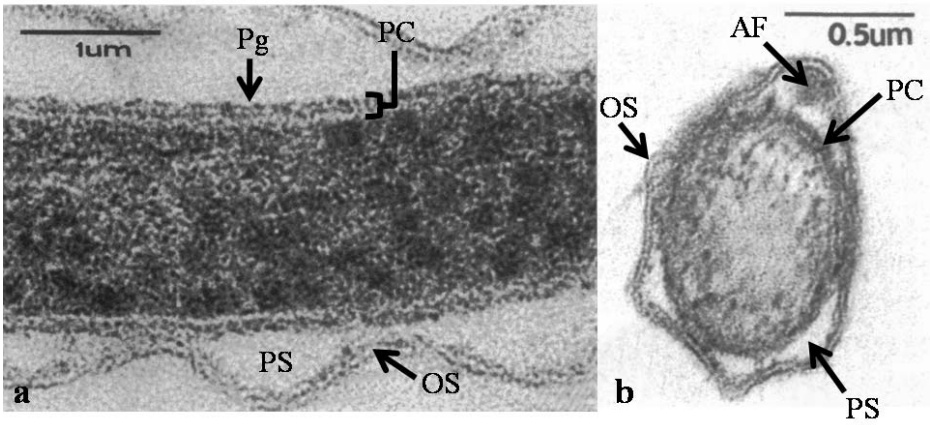


Figure 5. Electron micrograph of thin section of the *L. interrogans* shows (a) longitudinal section and (b) transverse section showing the outer sheath (OS), periplasmic space (PS), peptidiglycan cell wall (Pg) and Protoplasmic cylinder (PC) (Adapted from Holt, 1978).

The central core of the axial filament was composed of a family of FlaB proteins which showed the sequence similar to the flagellin (Wolgemuth *et al*., 2006), arranged in linear coil or globular form and surrounded by outer sheath (Faine *et al*., 1999). Amino acid analysis of axial filament revealed chemical component very similar to bacterial flagella (Holt, 1978), were composed of the protein and amino acid (Nauman *et al*., 1969). They were enriching with aspartic acid, glutamic acid, alanine, leucine and glycine, but they lacked tyrosine, tryptophan, and cysteine.

The protoplasmic cylinder has two parts, cell wall and cytoplasmic membrane (inner membrane) (Holt, 1978). It was tubular appearance, was proteinaceous and consisted of fibrils. The cell wall of protoplasmic cylinder contained the similar chemical composition, peptidoglycan complex to the cell wall of gram-negative bacteria (Holt, 1978). The chemical analysis of peptidoglycan presented muramic acid,

glucosamine (Holt, 1978), alanine, and glutamic acid which acted as major amino sugar components.

The inner membrane (cytoplasmic membrane) was made of peptidoglycan complex (Faine *et al*., 1999) and fibrils. The fibril contained bundles of fine fibrils that in some way associated with the protoplasmic cylinder (Holt, 1978).

Clinical features

The symptoms of leptospirosis begin at about 4 days to 4 weeks after exposure to *L. interrogans* (Rubin *et al*., 1999) and the incubation period was 10 days average (Nester *et al*., 2001). In mild form, leptospirosis may present as an influenza-like illness with headache and myalgia. These symptoms are presented in early phase of leptospirosis. Severe leptospirosis is characterized by jaundice, renal dysfunction, and haemorrhagic diathesis which are referred to Weil's syndrome (Dutta *et al*., 2005; Vieira *et al*., 2002; Ko, 1999). In general clinical manifestation of leptospirosis can be divided into two clinically recognizable syndromes. The most common syndrome is anicteric leptospirosis, 85% to 90% of patient present mild febrile illness. There are two clearly defined stages in anicteric leptospirosis; the septicemic stage or acute phase lasting about a week followed by immune phase, characterized by antibodies production and excretion of leptospires in the urine. Five percent to ten percent are severely ill with jaundice and more serious cases are potentially fatal. This stage is icteric leptospirosis (Erdinc 2005; Dutta *et al*., 2005, Sambasiva, 2003; Levett, 2001).Both anicteric and icteric leptospirosis may follow a biphasic course (Dutta *et al*., 2005).

Anicteric leptospirosis

The incubation period for leptospirosis is 2 to 20 days usually 7 to 12 days (Sambasiva, 2003). The onset of anicteric leptospirosis is abrupt and is characterized by fever, chill with rigors, headache, myalgia, nausea, vomiting, skin rash, conjunctival suffusion and prostration (Sambasiva, 2003, Dutta *et al*., 2005; Levett, 2001). Furthermore, Spicher *et al*. (2007) founded pancreatitis (acute hyperglycemia, insulin requirement, elevated lipase and amylase level) associated with anicteric leptospirosis.

The septicemic (or first) phase lasts 3 to 7 days. Fever is high and may reach a peak of 40˚C. Headache is intense, unremitting and possibly throbbing. Abdominal pain, anorexia, nausea and vomiting occur in the most patients. Conjunctival suffusion is characteristic and usually appears on the third or fourth day and is most common finding in absence of purulent discharge (Sambasiva, 2003; Pavia, 1994; Dutta *et al*., 2005).

Myalgia usually involves the muscle of calf, abdomen and paraspinal region. When present in the neck, myalgias may cause nuchal rigidity reminiscent of meningitis. In the abdomen, myalgia may mimic acute abdomen and leading to confusion with surgical intra-abdominal emergency. The characteristics of skin seen in mild leptospirosis include transient urticarial, macular or maculopapular and erythematous or purpuric rash (Dutta *et al*., 2005). In addition, the patients may show signs including splenomegaly, lymphadenopathy and hepatomegaly. Leptospires can be isolated from blood, cerebrospinal fluid and tissue. The symptoms are prominent for 4 to 7 days during the septicemic stage, at which time defervescence due to lysis occurs (Sambasiva, 2003)

The immune (or second) phase of anicteric leptospirosis is proceeded by leptospiruria (develops and persists for 1 to 3 weeks) and correlates with the appearance of IgM antibodies in the serum (Dutta *et al*., 2005). Fever, headache and vomiting are less severe at the onset of the immune phase than during the septicemic stage. The duration of the immune phase ranges from 4 to 30 days, and the leptospires disappearance from the blood and the CSF after the first day of this phase (Sambasiva, 2003). The leptospires now establish in the glomeruli of the kidney and are eliminated from all sites in the host except eyes and may be brain. They may persist for weeks or months. Moreover, fever and earlier constitutional symptom in some patients may be recur and have sign of meningitis, such as headache and photophobia. Central nervous system involvement in leptospirosis is most commonly occurring as aseptic meningitis. Complications, such as uveitis, optic neuritis, iridocyclitis, chorioretinitis, and peripheral neuropathy occur frequently in this phase (Dutta *et al*., 2005).

Icteric leptospirosis

Icteric leptospirosis or Weil's syndrome is more severe disease and persistent of high fever and jaundice. This is usually associated with hepatic dysfunction, renal

failure, vascular dysfunction, haemorrhage and multiorgans failure. Haemorrhage can occur as petichial, purpura and gastrointestinal haemorrhage. Multiorgans failure is associated with a very high mortality (Levett 2001, Sambasiva 2003; Dutta *et al*., 2005; Vaiphei *et al*., 2007).

In icteric leptospirosis can divided into two phases as in anicteric leptospirosis. During the leptospiraemic phase of icteric leptospirosis, the symptoms do not suggest the leptospirosis until the third to seventh day of illness, when development of azotaemia and jaundice occur. The biphasic course of the disease is obscured by persistent fever, jaundice and azotaemia. Jaundice appears, but there is no evidence of destructive of hepatocellular. Hepatic dysfunction occurs, but it resolves and it is rarely the cause of death. Hypoprothrombinemia occurs in few patients and responds to administration of Vitamin K. Serum bilirubin levels peak within seven days and the increase persists for a few days to several weeks. Renal involvement is common in both anicteric and icteric leptospirosis, but symptoms are present only in patients with icteric disease. Oliguria, anuria, and azotemia are commonly occur during the second week of illness, but may appear as early as 3 to 4 days after onset. Hypotension due to vascular damage occurs only in patients with icteric leptospirosis, haemorrhage occurs only in severe cases. In addition, laboratory tests showed abnormalities including thrombocytopenia, anemia, leucocytosis with neutrophilia and an increase in the level of creatinine phosphokinase (Sambasiva, 2003).

Pathology

Leptospirosis is performed by the vasculitis development, endothelial damage as well as inflammatory infiltration including monocytic cells, plasma cells, histiocytes and neutrophils. The gross examination demonstrated common petechial haemorrhages and may be extensive. In addition, organs are often discolored due to the degree of icterus. The histopathology showed most mark in the liver, kidney, heart and lung; however, the other organs may be affected according to severity of the infection (Levett, 2001).

Liver

The histopathology of the liver showed the infiltration of neutrophils in the region of the Glisson’s capsules. Hepatocytes showed eosinophilic granular cytoplasm and

pyknotic nucleus (Zollinger *et al*., 1971). The liver cell plates were disorganization and showed degenerative changes to mild interstitial edema and vascular congestion (Arean, 1962; Higgins and Cousineau, 1976; Alves *et al*., 1987). A massive destruction of extravascular red blood cells liberated by the haemorrhage diathesis, appeared to be the main cause of jaundice (Higgins and Cousineau, 1976). The hepatic cells showed enlarge with clear cytoplasm and multinucleated as well as multifocal necrosis (Arean 1962; De Brito *et al*, 1967; Szeredi and Haake, 2006). Moderate bile pigment was noted in the cytoplasm of liver cells. Kuffer's cells were hyperplasia and erythrophagocytosis. The sinusoids were congested and there were diffused or focal haemorrhages. The space of Disse showed distention by clear fluid and contained increased numbers of mononuclear cells, lymphocytes and irregular acidophilic structures (Arean 1962). Saglam *et al*. (2008) and De Brito *et al*. (2006) showed leptospiral antigens deposited in the cytoplasm of macrophages in the portal regions and hepatocytes. Additionally, leptospiral wavy form and large granules were seen in the cytoplasm of macrophages, endothelial cells and hepatocyte (Szeredi and Haake, 2006). Portal areas showed mild inflammatory infiltration made up of lymphocytes and macrophages (De Brito *et al*., 1967; Alves *et al*, 1987). Immunoperoxidase staining demonstrated leptospiral antigens on the endothelial lining of the dilated portal venules (Alves *et al*., 1987).

The histochemistry of the liver showed disappearance of glycogen granules in the hepatocytes. Alkaline phosphatase activity was generally increased. Acid phosphatase activity was more marked in Kupffer's cell. Esterase activity showed slight deviation from the normal. In most of the cases a diminution of the enzyme activity was seen at the centrolobular region (De Brito *et al*., 1967).

The ultrastructures of the infected liver showed enlargement of Kupffer's cells, with irregular shape and with many irregular dense bodies in the cytoplasm. Some of them, which have an irregular shape and a light central area are probably lipofuscin granules. The hepatocytes with definite lesions were found throughout the hepatic lobule. Microvilli of the hepatic cells are edematous, irregular, and less electron dense free surface. Some space of Disse's contained altered organelles and cell debris. The attachments between of the liver cells were usually preserved. One of the most remarkable and frequent lesion was total or partial disappearance of the microvilli of

bile ductules. Severe cases showed marked glycogen depletion and a predominance of smooth over rough reticulum. The mitochondrial changes were more intense in severe cases. However, many intact mitochondria were seen in the majority of the hepatic cells (De Brito *et al*., 1966; De Brito *et al*., 1967).

Kidney

The gross appearance of the cattle kidneys showed numerous and grayish-white focal lesion mainly located in the cortex, and occasionally reaching the outer medulla (Yener *et al*., 2001). The kidney of infected human demonstrated enlarged, boggy, with brownish yellow color and somewhat pale consistency. The capsules showed smooth shiny surfaces studded with petechial haemorrhages or containing large haemorrhages. The cut surfaces were edematous, glistening, and bulged past the capsule. Scattered petechial were seen in the cortex, while the medulla appeared congested and bluish red (Arean, 1962).

The histopathology of the biopsy kidney from human leptospirosis showed acute interstitial nephritis in the form of interstitial inflammatory cell infiltrate containing plasma cells, lymphocytes, macrophages and eosinophils (Salkade *et al*., 2005). The neutrophils were rarely seen but sometime they formed small clumps around the parietal layer of Bowman's capsule (Penna *et al*., 1963). In addition, one biopsy kidney showed interstitial edema and tubular dilatation lined by flattened cells with basophilic cytoplasm and prominent nuclei (Alves *et al*., 1987). The epithelial cells lining distal convoluted tubule, ascending limb of Henle's loop and collecting tubules were damaged (Arean, 1962). The proximal tubular cell showed hyaline droplets (De Brito *et al*., 1966). Moreover, brush border of proximal tubular cells disappeared and the differentiation between proximal and distal tubules sometime was quite difficult (Penna *et al*., 1963). The histopathology of animal kidney demonstrated similar to these of the human leptospirosis. For instance, in the horse, the interstitial edema, fibrosis and orange to brown pigments were observed (Hodgin *et al*., 1989). In the cattle, focal or diffuse mononuclear cell infiltration in the interstitial tissue of the cortex, outer medulla (Yener *et al*., 2001) and around the renal tubules was founded (Nally *et al*., 2004). The small particles of haemosiderin were demonstrated in the epithelial cells and macrophages (Yener *et al*., 2001). In the canine leptospirosis, some sections of the kidney showed inflammatory foci located in the renal pelvis

(Wild *et al*., 2002). Pilakasiri *et al*. (2001) demonstrated some congestion and swelling of the glomerular tuft in infected hamsters. The glomeruli showed many pathological changes, including haemorrhage in the glomerular tuft and urinary space as well as inflammatory cell infiltrations.

The immunohistochemical staining of the kidney sections showed the typical wavy forms of leptospires in the cytoplasm of different cell types such as endothelial cells, tubular epithelial cells of the kidney and extracellular in the connective tissues, in the lumen of blood vessels in various fetal organs of equine (Szeredi and Haake, 2006), and in the lumen of renal tubules of the equine and fatal human leptospirosis, (Szeredi and Haake, 2006; De Brito *et al*., 2006). Moreover, in the lumen of renal tubules demonstrated dense colonization of leptospires by Wartin-Starry stain (de Faria *et al*., 2007). The LipL41 was observed in one dog and was associated with intact organisms attached to the microvilli surface of a proximal convoluted tubule (Wild *et al*., 2002) as well as leptospires were seen in the lumen of renal tubule (da Silva *et al*., 2005). In addition, macrophages in foci of interstitial nephritis contained intense staining for leptospiral LPS antigen (Wild *et al*., 2002). In the aborted sheep, leptospiral antigen deposited in the cytoplasm of epithelial cells of renal pelvis, epithelial cells of cortical and medullar tubules, and macrophages of intertubular region (Saglam *et al*., 2008). The phagocytosed antigen was also observed in the cytoplasm of syncytial and multinucleated giant cells (Yener *et al*., 2001).

The electron microscopy showed the lesions mainly in injured tubular cells being throughout the nephron but predominant in the proximal convoluted tubules. The cells were edema and protrude into the lumen. The mitochondria were enlarged and swollen and the dilatations of the cisternnae of the smooth endoplasmic reticum were also found. Golgi complexs were usually unaltered. Furthermore, at the late phase of the disease was characterized by the focal partial disappearance of the microvilli of the proximal tubular cells. The interstitium was edema and capillaries dilatation was showed in this phase. In addition, endothelial cells showed decrease in the relative density of the cytoplasmic ground substance and reduced concentration of the ribonuclein granules and organells. The disjunction of endothelial cells were exhibited (De Brito *et al*., 1965; De Brito *et al*., 1966; De Brito *et al*., 1967; Ooi *et al*., 1972; De Brito *et al*., 1979).

Heart

Leptospirosis can cause myocarditis (Salkade *et al*., 2005). A transthoracic echocardiogram showed a mildly dilated left ventricle and mildly dilatation of right ventricle with moderate depressed contractility (O' Leary *et al*., 2004). Gross examination showed petechiae in the epicardium, along the coronary sulcus and in the endocardium of the left ventricle (Arean, 1962). The histopathology of heart of the patients who has been leptospirosis showed myocarditis with moderate infiltration by lymphocytes and monocytes (Arean, 1962; Salkade *et al*., 2005). In addition, the mesothelial cells were desquamation and replaced by irregular bands of fibrin. The myocardial fiber exhibited hyaline or granular degeneration, with clumping of sarcoplasm and intense acidophilia (Arean, 1962). Necrosis foci of myocardial were seen (Arean, 1962; Salkade *et al*., 2005) and rarely founded focal endocarditis of the aortic valves. Moreover, the observation showed rather marked infiltration of lymphocytes, plasma cells and a few neutrophils into the aortic adventitia, chiefly about vasa vasarum and extension into the outer media (Arean, 1962). In the infected hamsters showed histopathology of heart similar to Arean (1962) that autopsid the patients who died by leptospirosis, including degenerative change of cardiac muscle cell, congestion and dilatation of cardiac blood vessels. Furthermore, there were many haemorrhagic area and interstitial edema on the cardiac sections. Some cardiac muscle cells showed necrosis surrounded by numerous inflammatory cells (Muensoongnoen *et al*., 2006).

Lung

The incidence of pulmonary involvement in leptospirosis varies, but ranges from 20% to 70%. Cough, haemoptysis with blood stained sputum and dypsnea are the most common pulmonary symptom (Thammakumpee *et al*, 2005; Chawalparit *et al*, 2007; Shenoy *et al*, 2006). Some patients showed pleuritic chest pain (García *et al*, 2000).

The gross necropsy of fatal human leptospirosis revealed dark red and plum coloured on the lung (Salkade *et al*., 2005). Petechial were observed on the pleural surfaces and were more prominent in the hilar region and interlobular fissures. The tracheobronchial tree was studded with petechial or showed diffuse haemorrhages and chiefly in the posterior wall of the trachea (Arean, 1962). The histopathology showed pulmonary congestion, intra-alveolar haemorrhage, hyaline membrane and pulmonary

edema indicative of a predominant acute respiratory syndrome (Arean, 1962; Salkade *et al*., 2005). The leptospiral antigen was detected on the luminal surface of the endothelium and in the cytoplasm of the endothelial cells of septal capillaries in the patients (Nicodemo *et al*., 1997). Saglam *et al*. (2008) showed leptospiral antigen located in the cytoplasm of macrophages in interalveolar and interlobular septum. The electron microscopy showed the increase in number of pinocytotic vesicles, presence of giant dense bodies in the cytoplasm of these cells. The mitochondria showed few changes, with mild-to-moderate crista fragmentation. Platelets were identified in the lumen of septal capillaries and frequently adhering to endothelial cells. The electron dense amorphous material interposed between the surfaces of the endothelial cells and platelets were observed by Nicodemo *et al*., (1997)

In experimental study of the hamsters induced leptospirosis showed distinct massive area of haemorrhage on the surface of the lung and peritoneal surface (Praditpornsilpa *et al*, 2006) that was similar to the fatal human leptospirosis. The pulmonary sections showed alveolar and interalveolar capillaries distended and engorged with red blood cells and inflammatory cells especially polymorphonuclear cells. Inter-alveolar septum was thickened and consisted of an increased number of neutrophils, lymphocytes, plasma cells, pulmonary macrophages and brown haemosiderin granules (van de Ing and Hartman, 1986; Pilakasiri *et al*., 2001; Praditpornsilpa *et al*., 2006). In addition, Pilakasiri *et al*. (2001) demonstrated dilatation of alveoli which contained a homogeneous substance. Praditpornsilpa *et al*. (2006) showed stagnation and cytoadherence of the inflammatory cells to the endothelial walls of the arterioles. The histopathology of lung of the guinea pig induced leptospirosis showed the vacuolization of the endothelial cells. The intravascular fibrinoid organization was constant finding and free endothelial cells were seen in the lumen of vessels (Higgins and Cousineau, 1977).

The ultrastructure of infected lung showed bleb formation of alveolar epithelium and endothelium of capillary. Infected lung appeared to contain leucocytes and septal cells. The cytoplasm of the alveolar epithelial cells appeared swollen and protruded into the alveoli (Miller *et al*., 1974). The pulmonary capillary showed endothelial cell swollen and vacuolated cytoplasm (De Brito *et al*., 1979; Nally *et al*., 2004) with dilated endoplasmic reticulum and enlarged mitochondria. Platelet and leucocyte

thrombi, as well as capillary packed with red blood cells were demonstrated. Moreover, in severely damaged vessels the desquamation of endothelial cell from basement membrane and opened disjunctions of the cell were observed (De Brito *et al*., 1979).

Adrenal gland

Gross observation of adrenal gland showed haemorrhagic infiltration in the periadrenal fat and the medulla. Scattered petechial were noted in the cortical (Arean, 1962). Histopathologically there were congestion and multiple foci of cortical haemorrhages and exhibited diffused haemorrhage of the medulla (Arean, 1962; Salkade *et al*., 2005). In addition, the medulla showed disorganization of normal structure. Interstitial inflammation was characterized by focal of lymphocytes, neutrophils and plasma cells (Arean 1962). Szeredi and Haake, (2006) founded leptospiral antigens on epithelial cell in the cortex of the adrenal gland.

Pancreas

Pancreatis caused by leptospirosis was rare complication and only a few cases had been reported (Kaya *et al*., 2005; Spichler *et al*., 2007). The patients presented jaundice and epigastric pain was the main manifestation of pancreatic involvement (Daher *et al*., 2003; Kaya *et al*., 2005). The laboratorial diagnosis found high sera level of lipase and amylase, which are more specific for pancreatitis (Casella *et al*., 2000; Kaya *et al*., 2005; Spichler *et al*., 2007). Histopathology of pancreas finding were edema, mild inflammatory infiltration of lymphocytes, haemorrhage, congestion (Daher *et al*., 2003; Arean, 1962), fat necrosis and calcification (Daher *et al*., 2003).

Gallbladder

Leptospirosis could mimic the clinical symptoms of acute cholecystitis (Guarner *et al*., 2001; Kaya *et al*., 2005). Gross examination of gallbladders showed thickened walls with smooth serosal surface and bile stained mucosa. Histopathology demonstrated the edema and sparse foci of mononuclear cell in the submucosa and around some serosal vessels. In addition, immunohistochemistry stains for *Leptospira* showed small foci of granular and filamentous antigen staining in vessel walls and occasionally in the submucosa. Antigen staining was more abundant in the gallbladder from the male patient (Guarner *et al*., 2001).

Spleen

In 1962, Arean studied fatal human leptospirosis by necropsy. The histopathology showed congestion, scattered haemorrhage in the tissue of spleen. He founded hyperplasia of reticuloendothelial cells, erythrophagocytosis and increased of inflammatory cell such as neutrophils and plasma cell. In some case, eosinophils were observed. van der Ingh and Hartman (1986) demonstrated lymphoid hyperplasia with germinal centre formation and a plasma cellular reaction. In the study of Muensoongnoen *et al*. (2006) in hamsters' model, they showed the histopathology of the spleen which was like these of Arean (1962). The spleen of hamsters showed the degenerative change of the cell of the splenic cord and demonstrated brown hemosiderin granules which were the result of hemolysis of red blood cells in dilated splenic sinusoids and in the cytoplasm of macrophage (Nally *et al*., 2004). The immunohistochemical study showed leptospiral antigens in the cytoplasm of macrophages throughout the parenchymal tissue (Saglam *et al*., 2007).

Central nervous system

In the fatal human leptospirosis showed abnormality of central nervous system, but this abnormality are a rare event. Arean (1962) observed the engorged cerebral blood vessel, slightly green brain surface and focal perivascular lymphocytic infiltrations involving the white matter of the cerebrum, cerebellum, medulla, spinal cord as well as small and medium sized blood vessels (Panicker *et al*., 2001). The perivascular haemorrhages in human brain were observed by Arean (1962) and Theilen *et al*., (2002). In addition, the choroid plexus haemorrhage was observed (Arean, 1962). An intracranial haemorrhage arises as results of hypoprothrom-binaemia, thrombocytopenia and vasculitis (Panicker *et al*., 2001).

Peripheral nerve

Electromyography revealed marked fibrillation and severely reduced motor unit recruitment in the right anterior tibial muscle, indicating acute denervation of the right fibula nerve (de Souza *et al*., 2006). Moreover, the sections of sciatic nerve were obtained scattered haemorrhages, with slight disruption of nerve fibers (Arean, 1962).

Eye

The ocular manifestation of severe leptospirosis was noted in early reports. Conjunctival suffusion was seen in patient leptospirosis (Levett, 2001). Arean (1962)

reported one leptospirosis patient developed clinical signs of optic nerve atrophy. Horses infected with leptospires presented several clinical disorders. Acute sign observed in equine including blepharospasm, photophobia, lacrimation, myosis, corneal edema, vascularisation, aqueous flare and hypopyon. In addition, the inflammatory process may be lead to cataract, iris atrophy, retinal detachment, lens luxation and corneal opacity (Lucchesi *et al*., 2002).

Skeletal muscle

One of several clinical manifestations of was myalgia. The symptom was presented by Ding *et al*., 2001; Salkade *et al*., 2005 and Vieira *et al*., 2006. Blood analysis showed high serum myoglobin due to severe rhabdomyolysis (Turhan *et al*, 2006). Tenderness of bilateral calf muscle was observed in patient by Ding *et al*., (2001). In addition, haemorrhages were observed in the majority of the cases of Arean (1962). These were most common in the pectoralis, intercostals, psoas muscles and less often in the diaphragm, anterior abdominal wall, upper arm and thigh. Evident foci of grayish discoloration in the musculature of the anterior abdominal wall which corresponded to the sites of muscle necrosis were displayed.

Histologically, vacuolation to complete degeneration of the sarcoplasm, involving isolated muscle fibers or portions of one fiber with intervening normal segments were observed. The most severely affected fibers exhibited hyaline or granular degeneration of the sarcoplasm, with loss of longitudinal, cross striations and disruption of the sarcolemmal membrane. There were hypertrophy and hyperplasia of the sarcolemmal nuclei and infiltration by histiocytes, lymphocytes and neutrophils. Interstitial hemorrhages were associated with some, but not all, degenerative foci. Perivascular monocytic and lymphocytic infiltrations were seen in some of the affected areas. Some of these characteristics were observed in the dog, rat, hamster and guinea pig (Laurain, 1955). Electron microscopy of vessels in the diaphragm showed the swollen endothelial cell, endoplasmic reticulum dilatation, enlarged mitochondria, and disjunction of endothelial cell. Moreover, the endothelial cells showed bleb formation and sloughing from basement membrane (De Brito *et al*., 1979). In the leg, gastrocnemius muscle demonstrated dilation and congestion of blood vessels and group of inflammatory cells in the perimysium surrounding the muscle bundles. Furthermore, swelling, loss of cross striation, vacuolation and degenerative change of

muscle fiber were observed by Silva *et al*. (1980); Lecour *et al*. (1989) and Pilikasiri *et al*. (2001). In the hamstring muscle showed dilatation and congestion of blood vessel, necrosis of some muscle fiber and inflammatory cell infiltration. The necrotic area showed homogeneous eosinophilic substances replacing the degenerative change of muscle fiber (Pilikasiri *et al*., 2001).

Bone marrow

Bone marrow biopsy showed profound erythroid hypoplasia with exclusive predominance of lipocytes and revealed hypoplastic bone marrow with extremely low number of megakaryocytes (Somers *et al*., 2003; Stefos *et al*., 2005). In addition, the sections of bone marrow showed hyperplasia of granulocytic elements with numerous metamyelocytes (Arean, 1962).

Gastro-intestinal tract

Haemorrhages were seen in various organs, including the gastro-intestinal tract (Salkade *et al*., 2005). Arean (1962) demonstrated haemorrhages in the mucosa of the mouth, gums and blood clots in the nasal cavities in the autopsy fatal human leptospirosis. One patient exhibited haemorrhagic suffusion of the pharynx and esophagus as well as the mucosa of the gastroenteric tract showed the ulceration at the cardio-esophageal region. Histologically there were hemorrhages, isolated collections of plasmocytes and lymphocytes, and interstitial edema especially in the mucosa and submucosa. The large intestine showed apoptotic body in the overlying epithelium and an increased number of foamy macrophages in the lamina propria (Nally *et al*., 2004). Peyer's patches showed reticuloendothelial cells hyperplasia with erythrophagocytosis, disorganization of normal architecture and scattered hemorrhages (Arean, 1962).

Ureter and urinary bladder

The mucosa of the ureter and urinary bladder showed scattered petechial with foci of perivascular inflammation in the muscular layer (Arean, 1962).

Skin

The upper part of the dermis showed focal haemorrhages and perivascular lymphocytic and plasmocytic infiltration (Arean, 1962).

Other

The histopathology of the pituitary, thyroid and parathyroid glands showed foci of hemorrhage and lymphocytic infiltration in the interstitial were seen occasionally.

No other degenerative changes were observed (Arean, 1962). In natural case of equine abortion caused by leptospira, the placenta showed leptospiral antigens deposited (Szeredi and Haake, 2006).

Pathogenesis

The leptospires enter the body through the mucous membrane and abrasions or breaches of the surface integument, which may be trivial. No lesion develops at the site of entry of leptospires but the organisms multiply and spread throughout the body by blood circulation and penetrating all the tissue (Faine *et al*., 1999; Nester *et al*., 2001). After the host was infected with leptospires, they shows a diverse array of clinical manifestations ranging from subclinical infection to undifferentiated febrile illness to jaundice, renal failure, and potentially lethal pulmonary haemorrhage (Bharti *et al*., 2003). However, the mechanisms of leptospirosis caused by leptospires are not well understood (Levett, 2001). Pathogenic mechanisms of leptospirosis may be divided into direct effects by leptospires and host immune response to infection (Bharti *et al*., 2003).

1. Direct effects by leptospires

1.1 Toxin production

Endotoxic activity had been reported in several serovars. Lipopolysaccharides (LPS) of the leptospires exhibited activity in biological assays for endotoxin (Levett, 2001) and induced apoptosis of lymphocytes in mice (Lee *et al*., 2002). Hemolysin from several serovars such as serovars hardjo, ballum, pomona, and tarassovi had been characterized (Levett, 2001). Hemolysin was cytolytic toxin and classified into three categories base on the mechanism of the action on the membrane of the target cell, including enzyme, pore formation and surfactant (Lee *et al*., 2002). The pathogenic leptospires exhibited chemotaxis towards hemoglobin. Phospholipase C activity had been reported in serovar canicola (Levett, 2001). Hemolysin from serovar Lai had been reported to play an important role in pore formation. The gene that encoded this hemolysin was located upstream of the gene encoding sphingomyelin, which was another secretory protein that influenced hemolytic activity (Palaniappan *et al*., 2007).

1.2 Attachment of leptospires with host cells

Binding of the leptospires to host tissues was an importance step to establish the infection. The process of binding required the interaction between leptospiral surface structures and host component (Chirathaworn *et al*., 2007). The leptospires had the ability to attach to other eukaryotic cells including endothelial cell, fibroblasts and renal epithelial cells (Cinco *et al*., 2002). Twigg and Cox (1976) demonstrated colonies of the leptospires in proximal and distal convoluted tubules in all sections of the kidney. Especially, leptospires were found mainly in the proximal convoluted tubules and disappeared in the thin segment of Henle's loop. They were rarely seen in the thick segment of Henle's loop and distal convoluted tubules. The reason that leptospires were found mainly in the proximal tubules because in the mammals all glucose, most salt and some water were absorbed in proximal tubules and the remaining water was absorbed in the Henle's loop and distal convoluted tubules.

In addition, leptospires could bind to extracellular matrix proteins especially fibronectin, so they bound to fibronectin more than to collagen type IV and laminin (Chirathaworn *et al*., 2007). However, the effect of temperature on the attachment of leptospires to extracellular matrix was then examined. The number of attached leptospires increased gradually with increasing incubation temperature up to 30 ° C. The level of attachment was at 30 and 37 °C (Ito *et al*., 1987).

Leptospires were phagocytosed by macrophages. Inhibition of macrophages activity increased sensitivity to infection. Nevertheless, the outer envelop of leptospires had an antiphagocytic component (Levett, 2001). Moreover, leptospires demonstrated the ability to actively invade the monocyte-macrophage-like J774A.1 cells during the early stages of contact and induce apoptosis. The ability to invade and induce apoptosis of the macrophages were related to the pathogenicity of the leptospires and contributed to its ability to survive in the host and escape from the host immune response (Merien *et al*., 1997). The attachment pattern of leptospires displayed difference in different cell lines. When leptospires were incubated with J774A.1 cells, leptospiers adhered to one cell with both of their two ends. Besides, when they were incubated with Vero cells (African green monkey kidney fibroblasts) they attached to cells with only one of their end (Liu *et al*., 2007). LPS of leptospires

stimulated the adherence of neutrophils to endothelial cells and platelets which caused the aggregation and inducing the development of thrombocytopenia (Levett, 2001).

1.3 Surface protein of leptospires

The outer membrane of leptospires contained antigenic and virulent components such as lipoprotein, lipopolysacharide and peptidoglycan, as well as leptospiral endotoxin located on the outer membrane (Yang, 2007). These structures consisted mainly of glycolipoprotein (GLP) which could activate monocytes through receptors (Visith and Kearkiat, 2005). The leptospiral outer membrane protein activated nuclear transcription factor kappa B (NF-kB), activator protein-1, and downstream genes expression in medullary thick ascending limb cells of the kidney (Yang, 2007). In addition, the outer membrane protein up-regulate the expression of inducible nitric oxide synthase. This nitric oxide released by the medullary thick ascending limb of Henle's loops later generated peroxynitrite and caused renal injury. Stimulation of the NF-kB was responsible for the pathogenesis of glomerulonephritis and interstitial nephritis (Visith and Kearkiat, 2005). Furthermore, lipopolysaccharide 32 (LipL 32) was identified as the major pathogenic outer membrane lipoprotein and induced tubulointerstitial nephritis mediated gene expression in mouse proximal tubule cells (Yang, 2007).

A GLP fraction extracted from *Leptospira interrogans* contained a potent inhibitors which inhibited the Na, K-ATPase of the renal tubular epithelial cell (Younes-Ibrahim *et al*., 1997; Diament *et al*., 2002; Dorigatti *et al*., 2005; Burth *et al*., 2005) and was cytotoxic to the cultured mouse fibroblasts (Lee *et al*., 2002). The inhibition effect of GLP seemed too specific for the Na,-K-ATPase enzyme (Younes-Ibrahim *et al*., 1997). As a result, Na, K-ATPase was inhibited due to hypokalemia. This hypokalemia could cause myalgia, progressive quadriparesis, respiratory muscle weakness, dysphagia, tachypnoeic, flaccid hyporeflexic weakness abdominal distension with absent bowel sounds (Krishnan *et al*., 2003). In addition, GLP induced tumor necrosis factor alpha (TNF-α) from peripheral blood mononuclear cells. The TNF-α could be observed in the plasma of patients (Cinco *et al*., 1996; Diament *et al*., 2002; Dorigatti *et al*., 2002). The TNF-α induced monocytes to secrete cytokines such as IL-1, IL-6 and IL-8. These cytokines were essential in the control of infection in tissue lesions (Diament *et al*., 2002). The TNF-α was pro-inflammatory cytokine that

was released and induced the inflammatory process through the generation of several vasoactive and inflammatory mediators and adhesion molecules (Visith and Kearkiat, 2005).

The Th1 and Th2 lymphocytes were involved in immune mediated nephropathy (Visith and Kearkiat, 2005). The tissues penetration and invasion is presumably accomplished by a pair of axial filaments and release of hyaluronidase (Dutta *et al*., 2005). The LPS, outer membrane protein (OmpL1) and lipoprotein (LipL41) of pathogenic leptospires were demonstrated on the lumen of proximal convoluted tubules 10 and 28 days post infection. At 28 days post infection the renal interstitial tissues were present inflammatory cells. LPS and OmpL1 were also detected in the interstitial phagocytes. These data established that outer membrane components expressed during infection had roles in the induction and persistence of the interstitial nephritis (Barnett *et al*., 1999). Tian *et al*. (2006) demonstrated the outer membrane protein extracted from *Leptospira interrogans* serovar shermani could induced the increase of collagen type I and type IV production. As a result, extra cellular matrix accumulation in proximal tubular cells caused tubulointerstitial fibrosis. Also outer membrane protein induced early inflammation through a Toll-like receptor 2 (TLR2) pathway and increase of inflammatory cytokines and chemokines including inducible nitric oxide (iNOS), monocyte chemoattactant protein-1 (MCP-1) and TNF-α (Yang *et al*., 2000; Yang *et al*., 2006).

2. Host immune response to infection

The second stage of acute leptospirosis was referred to the immune phase. In this stage, the leptospires disappeared from the blood circulation but antibodies appeared. The immune mediated disease had been presented as one factor influencing the severity of the symptoms. In addition, the production of immune complexes leading to inflammation in the central nervous system had been postulated. The levels of immune complexes in the circulation were correlated with the severity of symptoms (Levett, 2001). In addition, glomerulonephritis with deposition of IgM in the mesangium indicated the humoral mechanism. The IgG, anti-cardiolipin antibodies, anti-neutrophil cytoplasmic antibodies (ANCA) and anti-platelet antibodies which were auto antibodies were detected (Visith and Kearkiat, 2005).

Diagnosis and Treatment

Diagnosis

Definitive diagnosis of leptospirosis depended on (1) isolation of leptospires, (2) serological tests, (3) detection of specific DNA (Dutta *et al*., 2005)

1. Isolation of leptospires

1.1 Blood

The organisms might be identified by dark field microscopy examination of the blood patients or by culture on a semisolid medium such as Ellinghausen-McCullough-Johnson- Harris (EMJH). If the blood were taken before the tenth day of illness, cultures took about 1-6 weeks to become positive (Dutta *et al*., 2005).

1.2 Urine

The leptospires might be isolated from the urine on the tenth day onwards and could be examined under the dark field microscope (Dutta *et al*., 2005).

2. Serological tests

2.1 Agglutination tests

Microscopic technique using live organisms and macroscopic technique using killed antigens became positive after 7-10 days of illness and peaked at 3-4 weeks. These might persist at high levels for many years. Thus, to make a diagnosis, a fourfold or greater rise in titer must be documented. The agglutination tests were cumbersome to perform and require trained personnel. The specific serovar was detected only by microscopic agglutination test (MAT) and culture isolation (Dutta *et al*., 2005).

2.2 Enzyme-Linked Immuno Sorbent Assay (ELISA)

IgM and slide agglutination tests (SAT) were also available. ELISA IgM and SAT were simple and sensitive tests which measure IgM antibodies. The IgM ELISA test was particularly useful in making an early diagnosis. However, Microscopic agglutination test (MAT) was the Gold Standard test but this method was complicated and less sensitive compared to new test like ELISA IgM and SAT (Dutta *et al*., 2005).

3. Detection of specific DNA

3.1 Polymerase Chain Reaction (PCR) test

PCR methods were sensitive, specific, positive in early disease, and could detect the leptospiral DNA in urine, blood, aqueous humor and cerebrospinal fluid

(CSF). At present, major disadvantage with these tests is specific to genus of leptospires, not specific to serovar (Dutta *et al*., 2005).

Treatment

The efficacy of the antibiotics in the treatment of leptospirosis had been debated. The leptospires with their typical bacterial cell walls and ribosomes are theoretically susceptible to a wide variety of antibiotics (Plank *et al*., 2000). Various antimicrobial drugs, including tetracyclines and penicillin, showed the antileptospiral activity. Penicillin was the drug of choice in severe leptospirosis and was especially effective if it was administered within first four days of illness. Total duration of therapy should be 10-14 days. Erythromycin and amoxycillin had been found effective in severe leptospirosis. Leptospirosis patient should be observed for evidence of renal failure and treated with hemodialysis if necessary. Patients with Weil's disease had hemorrhagic manifestation might require whole blood or platelet transfusion. Patients with multi-organs failure required to be observed in the intensive care unit. Besides, penicillin and doxycycline in a dosage of 100 mg twice daily for 7 days was effective in the treatment of mild and moderate leptospirosis. Effective of prophylaxis consisted of doxycycline 200 mg orally once weekly during the risk of exposure (Dutta *et al*., 2005). However, antibiotic treatment might be useless and immunomodulation might be the optimal approach (Pappas *et al*., 2006).

Normal histology of experimental organs

Liver

The liver is reddish brown organ that locates on the right side of the peritoneal cavity (Ham, 1969). The liver is enveloped by peritoneum which forms simple squamous epithelium covering over the dense irregular connective tissue (Glisson's capsule) and is composed of uniform parenchymal cells (hepatocytes) (Gartner and Hiatt, 2001). The hepatocytes are large polyhedral cells with round prominent nuclei with peripherally dispersed chromatin (Young and Heath, 2000). They are closely packed together to form anatomosing plates of liver cells, one to two cells in thickness (Gartner and Hiatt, 2001). The hepatocytes can take sugar from the blood and store it as the glycogen. They also take amino acids and build them into protein and can also

store fat and vitamins (Ham, 1969). The hepatocytes have important role in detoxification of various drugs and toxin (Young and Heath, 2000).

Hepatocyte surfaces (Figure 6)

Each hepatocyte processes three surfaces, including intercellular, canalicular and sinusoidal surfaces. The intercellular surface is the surface that each hepatocyte contacts with each other. Canalicular surface is the surface that faces the bile canaliculi. At this surface the hepatocyte has microvilli projecting into the bile canaliculi for increasing the surface area through which the bile can be secreted. The sinusoidal surface that faces the hepatic sinusoid also has microvilli which projects into the space of Disse (Gartner and Hiatt, 2001).

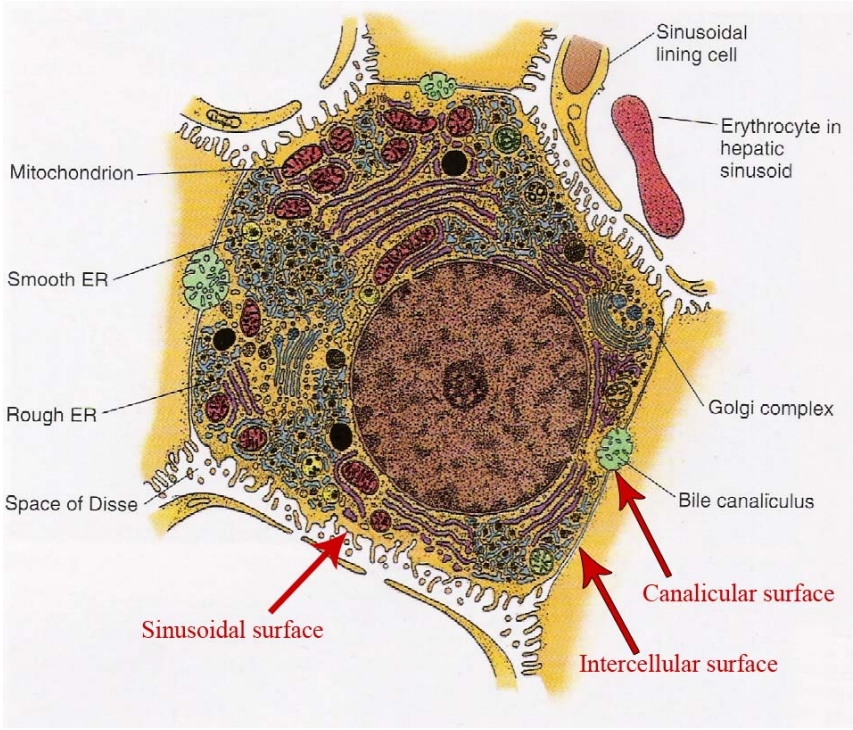


Figure 6 Diagram illustrating the hepatocyte surfaces (Adapted from Gartner and Hiatt, 2001).

Liver lobule (Figure 7)

The unit of hepatic parenchyma is classified into three types, including hepatic lobule, portal lobule and hepatic acinus.

Hepatic lobule is roughly hexagonal in shape with a central vein in the center. The portal tract is positioned at the angles of the hexagon. The blood from tributaries of the portal vein and branches of the hepatic artery in the portal tracts flows to the central vein (Young and Heath, 2000).

Portal lobule is defined as the triangular region whose center is the portal area and periphery is bounded by the imaginary line connecting the three surrounding central veins that form the tree apices of the triangle (Gartner and Hiatt, 2001). The portal lobule describes the bile draining from the surrounding hepatic parenchyma (Kierszenbaum, 2007).

Hepatic acinus is more accurate representation of the liver function. This acinus was a roughly berry-shaped unit of the liver parenchyma centered on portal tract. The acinus lined between two or more terminal hepatic venule and blood flow from the portal tracts through the sinusoid to the venules. It is divided into three zones. The zone I is closed to the portal tract and receives the most oxygenated blood, zone II is intermediate region and zone III is the farthest away and receives the least oxygen. Thus, the zone III is the most susceptible to ischemic injury (Young and Heath, 2000).

Hepatic sinusoid

The hepatic sinusoid is lined by endothelial cells that do not make contact with each other. Additionally, Kupffer cells are associated with sinusoidal lining cells (Gartner and Hiatt, 2001).

Perisinusoidal space (Space of Disse)

The perisinusoidal space separates sinusoidal lining cells from hepatocyte (Gartner and Hiatt 2001). This space contains type I, III and IV collagen fibers. Protein absorption and secretion take place across this narrow space (Kierszenbaum, 2007)

Blood supply of the liver

The liver takes blood supply by two blood vessels, including the portal vein and hepatic artery. The portal vein transports blood from digestive tract, pancreas and spleen. The hepatic artery supplies oxygenated blood to the liver by interlobar artery and interlobular artery pathway before reaching to the portal tract. The blood from the portal vein and hepatic artery mixed in the sinusoids of the liver lobule. Sinusoidal

blood converges to the central vein to form the sublobular vein and return to inferior venacava by the collecting vein and hepatic vein pathway (Kierszenbaum, 2007).

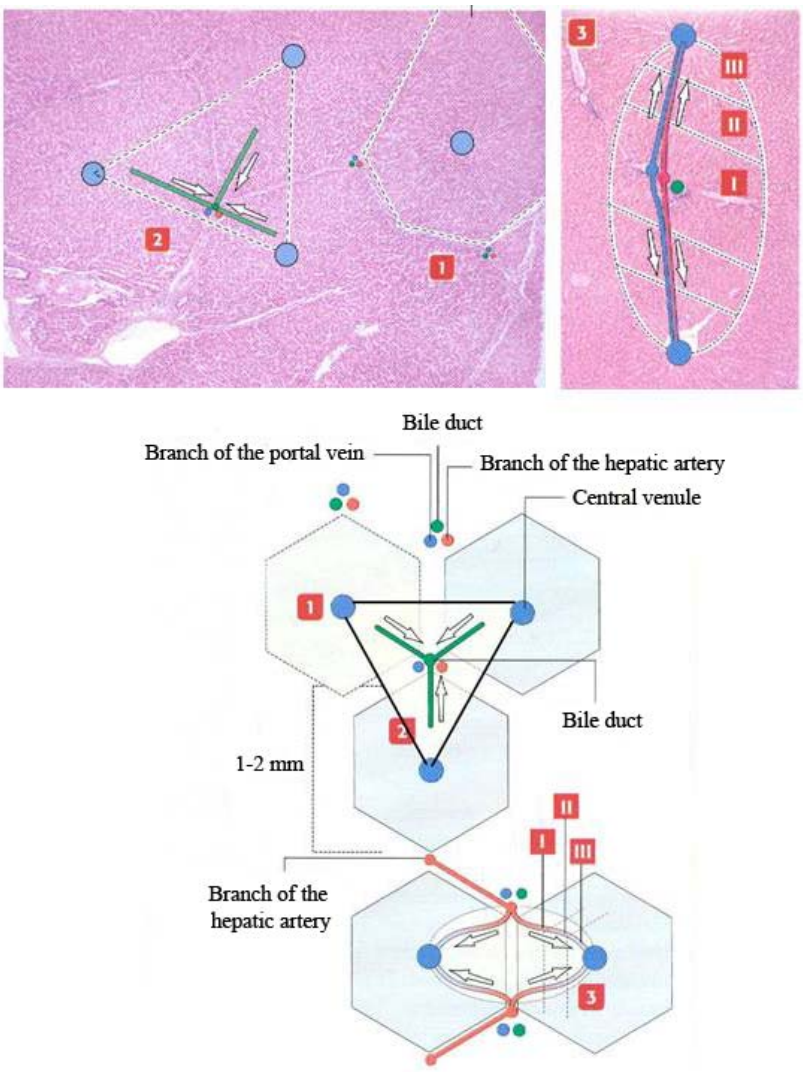


Figure 7 Diagram illustrating hepaticl lobule (1), portal lobule (2) and liver acinus (3) (Adapted from Kierszenbaum, 2007).

Spleen

The spleen is the largest lymphoid organ in the body. It locates in the peritoneum in the upper left quadrant of the abdominal cavity. The spleen has a convex surface and concave aspect known as the hilum. The capsule is dense irregulary fibroelastic connective tissue, occasionally is surrounded by simple squamous epithelium of the visceral peritoneum. The capsule is thickened at the hilum where the arteries, vein,

lymph vessels and accompanying nerve fibers enter. The histology of the spleen has three dimensional network of reticular fiber and associated reticular cells. The cut surface of the fresh spleen shows the gray area called white pulp which is surrounded by red area, red pulp (Gartner and Hiatt, 2001).

White pulp

The structure of the white pulp is composed of the periarterial lymphatic sheath (PALS), housing T cells, housing B cells and lymphoid nodule. These structures closely associated with the central artery. The central artery is surrounded with PALS of T lymphocyte. The PALS is enclosed within lymphoid nodules, which are composed of B cells and displace the central artery to the peripheral position. The white pulps are surrounded by the marginal zone that separates the white pulp from the red pulp. This zone is composed of plasma cells, T and B lymphocytes, macrophages and interdigitating dendritic cells. In addition, numerous small vascular channels, marginal sinuses are present in this zone (Gartner and Hiatt, 2001). Thus, this zone plays a major role in the immunological activity of the spleen (Junqueira and Carneiro, 2005).

Red pulp

The red pulps are composed of splenic sinuses and splenic cords (Figure 8).

Splenic sinus (Splenic sinusoid)

The splenic sinus is unusual in that it is fusiform shape resembling the barrel. This sinusoid is surrounded by reticular fibers that wrap around the sinusoid as individual, thin strands of thread and are perpendicular to the longitudinal axis of the sinusoids. The splenic sinusoid is coated by a discontinuous basal lamina (Gartner and Hiatt, 2001).

Splenic cords

The splenic cords are composed of loose network of reticular fibers whose interstices are permeated by extravasated blood. The reticular fiber is produced from the stellate reticular cells. The reticular cells and fibers support plasma cells, red blood cells and macrophages. Moreover, macrophages are particularly numerous in the vicinity of the sinusoids (Kierszenbaum, 2007).

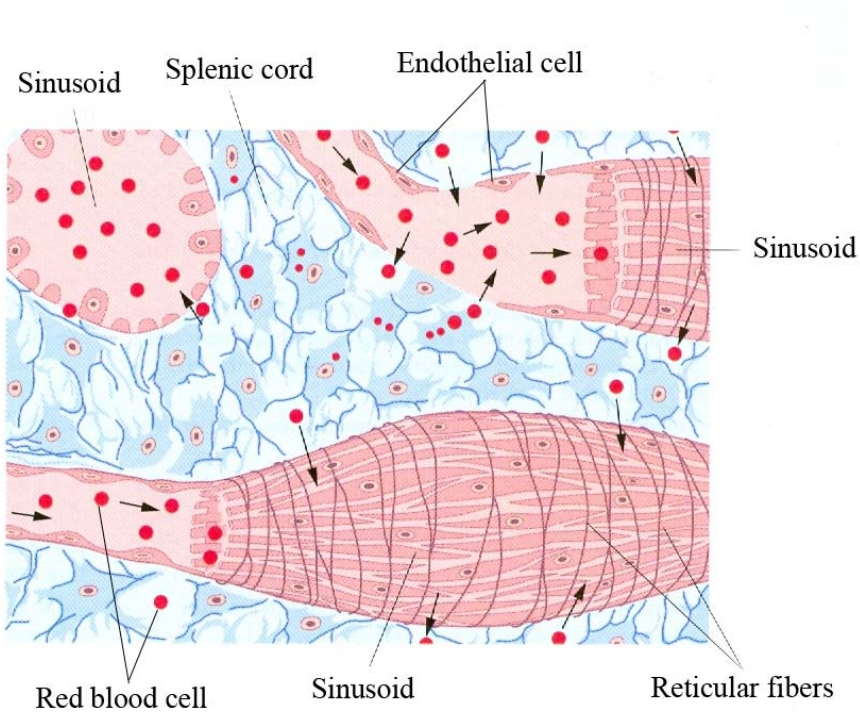


Figure 8 Diagram of the red pulp showing splenic sinusoid and splenic cords with reticular connective tissue. The reticular fibers form a three dimensional network in splenic pulp and surround the sinusoids. Spaces between endothelial cells at the sinusoid allow the passage of the blood cells. The arrows indicate the passage of the red blood cells. (Adapted from Junqueira and Carneiro, 2005).

Blood supply of the spleen

The splenic artery enters the hilum and give rise to trabecular arteries. The trabecular arteries distribute to the splenic pulp along the connective tissue trabeculae. They leave the trabeculae and become invested by a sheath of T cells formed a PALS and penetrate a lymphatic nodule. The blood vessel is designated the central artery and when it leaves the white pulp it becomes the penicillar artery. The terminal capillaries either drain directly into the spenic sinusoids (closes circulation) or terminate as open-ended vessel within the red pulp (open circulation). The spenic sinusoids drain to the pulp vein, to trabecular vein and then to spenic vein (Kierszenbaum, 2007).

Lung (Figure 9)

The lung is subdivided into two major portions, the conducting portion and the respiratory portion. The conducting portion conveys air from outside to the lung, consisting of the primary bronchi, secondary bronchi (lobar bronchi), tertiary bronchi (segmental bronchi), bronchiole and terminal bronchiole. The respiratory portion locates strictly within the lung and has the function in exchange of oxygen and carbondioxide, consisting of respiratory bronchiole, alveolar duct, alveolar sac and alveoli (Figure 9A) (Gartner and Hiatt 2001).

Bronchus

The bronchus continues from the trachea whose wall comprises the mucosa, muscular layer and hyaline cartilage. The mucosa is lined by the ciliated pseudostratified columnar epithelium with goblet cells. The connective tissue of the lamina propria contains numerous longitudinal elastic fiber (Geneser, 1986) and abundance of mucous and serous glands (Junqueira and Carneiro, 2005). The muscular layer forms the ring structure surrounding the mucosa and consists of densely paked bundle of smooth muscle cells (Geneser, 1986). The hyaline cartilages are the outermost layer and irregular in shape with collagenic connective tissue which is continuous with the perichondrium of the cartilages (Ham, 1969).

Bronchiole

The lumen of bronchiole is lined by the ciliated columnar epithelium with occasional goblet cells in the larger bronchioles and the simple cuboidal epithelium with occasional clara cells and no goblet cell in the smaller bronchioles. External to the mucosa is the lamina propria that contains no gland and is surrounded with loose meshwork of helically oriented smooth muscle layer. The smooth muscle layer is relatively thicker in the bronchioles than in the bronchi. The bronchioles have no hyaline cartilage in their wall (Gartner and Hiatt, 2001 and Geneser, 1986).

Terminal bronchiole

The terminal bronchioles are the most distal region of the conducting portion of the respiratory system. The epithelium of the terminal bronchioles are composed of one layer of cuboidal cells, some of which contain cilia and clara cells. The lamina propria is narrow and consisting of fibroelastic connective tissue and is surrounded by smooth muscle cells of one or two layer (Gartner and Hiatt, 2001 and Geneser, 1986).

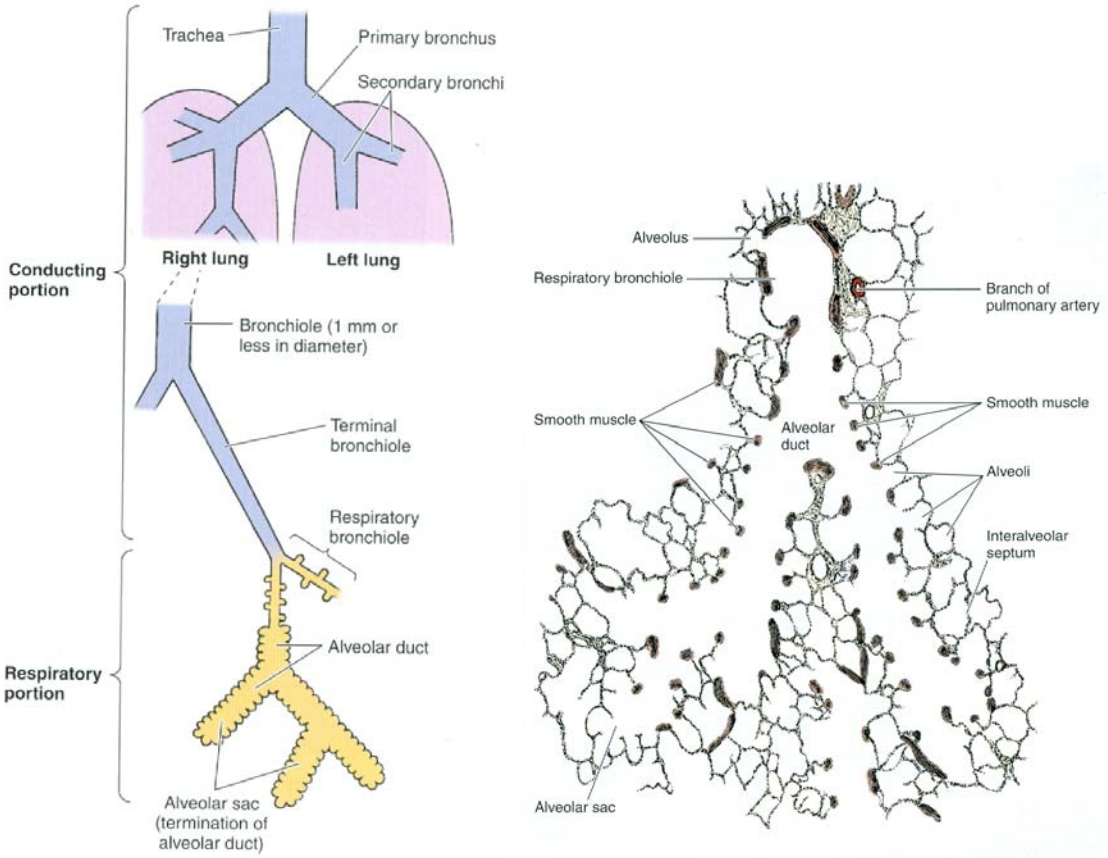


Figure 9 A, Diagram of the main divisions of the respiratory tract, consisting of the conducting portion and respiratory portion. B, Diagram of the portion of the respiratory part showing the respiratory bronchiole, alveolar duct and alveoli (Adapted from Junqueira and Carneiro, 2005).

Respiratory bronchiole

The respiratory bronchiole is the first region of the respiratory system where exchanges of gases can occur. The pouch-like structure known as alveoli first appears in the respiratory bronchiole (Figure 9B). They continue from terminal bronchiole and is lined by simple low columnar epithelium, consisting of ciliated cells and clara cells. This epithelium is surrounded by connective tissue, in which the well developed smooth muscle layer is embedded (Gartner and Hiatt, 2001 and Geneser, 1986).

Alveolar ducts, Atrium and Alveolar sac

Alveolar ducts are the tubes that arise from respiratory bronchiole and are merely linear arrangement of alveoli. The tubes are represented only by scattering groups of low columnar epithelial cells that line strand of smooth muscle embedded in connective tissue. The smooth muscle disappears at the termination of the alveolar duct. The end of each alveolar duct is atrium, which connects with two or more alveolar sacs. The alveolar sac is surrounded by alveoli (Figure 9B) (Gartner and Hiatt, 2001 and Geneser, 1986).

Alveolus

The alveolar wall is thin tissue septum separating two neighboring alveoli (Geneser, 1986). The region between adjacent alveoli is interalveolar septum. It is occupied by continuous capillaries, supplied by the pulmonary artery and drained by the pulmonary vein. The connective tissue of the interalveolar septum is rich with elastic fiber and reticular fiber (collagen type III). Wall of the alveoli are composed of pneumocyte type I and pneumocyte type II (Figure 9B) (Gartner and Hiatt, 2001).

Pneumocyte type I (Type I alveolar cell and squamous alveolar cell)

The pneumocytes type I are simple squamous epithelium. They occupy approximately 95% of the alveolar surface. They prevent the seepage of extracellular fluid into the alveolar lumen (Gartner and Hiatt, 2001).

Pneumocyte type II (Type II alveolar cells, Great alveolar cells and Septal cells).

The pneumocytes type II occupied about 5% of the alveolar surface. They are cuboidal cells and are interspersed among and form occluding junction with pneumocyte type I. They produce and secret pulmonary surfactant (Gartner and Hiatt, 2001).

Alveolar macrophage (Dust cell)

The alveolar macrophage phagocytoses particulate matter, such as dust and bacteria in the alveolar lumen. They migrate between pneumocyte type I and enter the lumen of the alveolus (Gartner and Hiatt, 2001).

Blood supply of the lung

The pulmonary arteries supply deoxygenates blood to the lung form right side of the heart. Because of these vessels follow the bronchial tubes into the lobules and become continuous with capillary. The blood in the capillary becomes oxygenated and

then drains into tributary of pulmonary vein. In addition, bronchial arteries, which are branches of the thoracic aorta brings nutrients and oxygen to the bronchial tree, interlobular septum and pleura of the lung (Gartner and Hiatt, 2001).

CHAPTER II
OBJECTIVES

The objective of this study was to determine the presence of leptospiral antigens in tissue sections of liver, spleen and lung of hamsters that infected with *Leptospira interrogans* serovar pyrogenes at different infectious duration by using immunoperoxidase staining technique. The results of this study will clarify

1. the progression of the disease.
2. the amount of the antigen observed corresponds to the severity of the infected organs as the infection time pass by.
3. where the antigen is located in the organ or tissue and it is located in/or nearby the histopathological area as well as inside or outside the parenchymal cells of the organs.

CHAPTER III
LITERATURE REVIEW

In 1979, De Brito and colleagues studied kidney, diaphragm and lung of guinea-pigs that were inoculated with *L. interrogans*, serovar icterrohaemorrhagiae by light and transmission electron microscopy (TEM). Light microscopy showed the pathological changes in those tissues, including focal areas of slight edema and haemorrhage. In the kidney, interstitial capillaries appeared congested in the vessel of the corticomedullary junction. In the kidney, the leptospira was observed in the endothelial junction of a renal venule. Endothelial swelling and vacuolization as well as the necrotic lesion in the peritubular capillaries were displayed. TEM demonstrated endothelial cells of capillary in the diaphragm were swollen and the open endothelial junctions were observed. The endoplasmic reticulum appeared dilated and mitochondria enlarged with a light matrix and a few cristae. Pericytes of the capillaries appeared shortened, with an irregular contour and electron-dense cytoplasm and nucleus. Moreover, red blood cells and fragments of leptospires were detected between the muscular fibers of the diaphragm near injured capillaries. In the lung, the swollen endothelium with dilated endoplasmic reticulum and enlarged mitochondria were observed. Many blebs were detected in the endothelial cell of lung capillary.

In 1987, Alves and colleagues studied fifteen samples of liver (nine from autopsies and six from biopsies) and nine samples of kidney (eight from autopsies and one from a biopsy) from leptospirosis patients by immunoperoxidase staining technique (antibody against *L.interrogans*, serovar icterrohaemorrhagiae). In the liver, the results showed leptospiral antigen depositing in all cases. Some appeared as either granular or thin thread-like filaments locating chiefly at the periphery of the portal tract, along the periportal sinusoidal lumina and the endothelial lining of dilated portal venules. Usually, leptospiral antigen was observed as a focal lining apparently on the luminal side of the vessel and the cytoplasm of endothelial cells. Some antigen was

revealed in the cytoplasm of Kupffer cells and some in the portal macrophages. In the kidney, it was revealed that the focal, thin, thread-like of leptospiral antigen were located in the interstitium of the cortex and outer medulla in some cases. Besides, the antigens were detected on the endothelium lining of dilated cortical capillaries and small vessels of the outer medulla, cases the endothelial lining of the arcuate arteries and in macrophages.

In 1997, Nicodemo and colleagues studied lung fragments from 12 patients which were collected immediately after death by light microscopy, TEM and by immunohistochemistry (antibody against *L. interrogans* serogroup icterohaemorrhagiae and serovar monymusky). Light microscopy showed edema of the intraalveolar septa in all cases and a slight-to-moderate inflammatory infiltration with a predominance of macrophages, lymphocytes and plasma cells. Ten cases exhibited endothelial tumefaction and nine cases showed alveolar haemorrhage. The leptospiral antigen was detected in eight cases by immunohistochemistry. The positive granular material was observed on the luminal surface of the endothelium and in the cytoplasm of the endothelial cell of septal capillaries and also in the filamentous form attaching to the endothelium of the septal capillaries. TEM demonstrated tumefaction of the cytoplasm of endothelial cells, increased numbers of pinocytotic vesicles and presence of giant dense bodies in the cytoplasm of these cells. The mitochondria showed few changes, with mild-to-moderate crista fragmentation. Platelets were identified in the lumen of septal capillaries in all cases and frequently adhering to endothelial cells.

In 2001, Pilakasiri and colleagues described the histopathology of the organs of hamsters infected with *L. interrogans* serovar pyrogenes by light microscopy. Infected kidney showed degenerative changes of the renal tubular cells and the glomerular tuft, congestion of the renal blood vessels, haemorrhage and inflammatory cell infiltration. The renal tubular cell showed vacuolar degeneration and nuclei were still intact with a vesicular appearance. The boundary of each cell was hard to distinguish. Some renal tubular cells lining the proximal tubules showed cellular swelling with slightly stained cytoplasm and varying degrees of necrosis. In addition, there were some changes in the distal tubules. There were dilatation of the tubular lumen, flattening of the epithelium and also necrosis. The histopathology of glomeruli showed the

inflammatory cell infiltration and haemorrhage in the glomerular tuft and the urinary space. The renal vasculature of both cortex and medulla was congested with red blood cells.

The lung sections in the early group of infection showed a small number of alveoli which were filled with red blood cells and inflammatory cells. The alveolar and interalveolar capillaries were distended and engorged with red blood cells. The alveolar septum was slightly thickening. The degree of severity was increased with the duration of infection. The infected liver showed enlarged and vacuolated hepatocytes being related to cloudy swelling of the hepatocytes. Vascular and sinusoidal congestion, prominent Kupffer cells, and inflammatory cell infiltration in the hepatic parenchyma and hepatic sinusoids were also demonstrated. The portal area showed a number of inflammatory cells. Hepatocellular necrosis was found scattering throughout the hepatic lobules which was a sign of hepatocellular damage and disorganization of the liver structure and function. In the gastrocnemius muscle showed the dilatation, congestion of blood vessels and a number of inflammatory cells in the perimysium surrounding the muscle bundle. In the hamstring muscle showed dilatation and congestion of blood vessels. Some muscle fibers showed necrosis and inflammatory cell infiltrations.

In 2002, da Silva and colleagues revealed clinicopathological features of four leptospirosis patients and three of them died. Lung of three dead patients were studied by light microscopic and immunoperoxidase technique (antibody against *L. interrogans* serovar copenhageni). Four patients appeared the severe pulmonary injury characterized by alveolar haemorrhage and acute respiratory failure. Leptospiral antigen in lung tissues was positive in all cases. Fine granular positive reactions corresponding to leptospires engulfed by macrophages were found in septa and alveoli.

In 2004, Nally and colleagues studied organs of guinea-pigs that injected with *L. interrogans* serovar copenhageni by light microscopy, TEM, immunohistochemistry, immunofluorescence and hematology. Gross autopsy revealed multi focal areas of haemorrhage on surfaces of the lungs and extensive haemorrhage on peritoneal surfaces. The haemorrhage also involved the intestinal mesenteric surfaces. The microscopic of the liver demonstrated hepatocyte necrosis and cellular discohesion.

Activated Kupffer cells were commonly seen in the sinusoids. Mild to moderate increases in number of monocytes and neutrophil were observed in portal tract. Silver and immunohistochemical staining confirmed the large number of leptospires in infected liver.

Infected kidneys were characterized by tubular necrosis. Inflammatory cells were present in and around the renal tubules. Silver and immunohistichemical staining demonstrated leptospiral antigen along the tubular basement membrane, between tubular cells, within the tubular lumens, within the interstitium and in some cases were present within glomeruli. Infected intestine showed focal mucosal haemorrhage and edema. Increase in number of foamy macrophages in the lamina propria and apoptotic bodies in the overlying epithelium was revealed in large intestine. Immunohistochemistry confirmed the presence of the large numbers of intact leptospires within the mucosal of the large and small intestine. Infected spleens showed reticuloendothelial system activation with increased hemophagocytosis. Activated histiocytes with expanded pale to foamy cytoplasm were observed predominantly within the red pulp. Immunohistochemistry confirmed the presence of large numbers of intact leptospires in the spleens. Infected lung demonstrated that the hemophagocytic histiocytes were seen in haemorrhagic alveolar spaces. Increase in numbers of neutrophils in capillaries was observed. Intact leptospires were only rarely detected by immunohistochemistry. Ultrastructural studies of lung demonstrated endothelial and epithelial injury. Immunofluorescent technique was performed on infected lung tissues and confirmed the presence of IgM, IgG, IgA and C3 along the alveolar basement membrane. Hematology and chemistry showed elevated fibrinogen level and decreased platelet.

In 2006, Muensoongnoen and colleagues showed histopathological changes of hearts and spleen of hamsters infected with *L. interrogans* serovar pyrogenes by light microscopy. The histopathology of infected heart showed degenerative changes of the cardiac muscle cells, haemorrhage and inflammatory cells infiltration. The boundary of each cell was hardly distinguishable. The vessels were dilated and congested. In addition, the cardiac sections showed many haemorrhagic areas and interstitial edema. Some cardiac muscle cells showed various degrees of necrosis that surrounded by numerous inflammatory cells. The infected spleens showed degenerative changes of

the splenic cord, congestion of the splenic sinusoid, haemorrhage and inflammatory cell infiltration. Small foci of cellular necrosis were found scattering throughout the splenic cord. Moreover, brown hemosiderin granules were seen in dilated splenic sinusoids and in the cytoplasm of macrophages. The haemorrhagic areas were scattering throughout the red pulp of the spleen.

In 2007, Chirathaworn and colleagues studied the binding of the pathogenic (serovar icterrohaemorrhagiae) and non-pathogenic leptospire (serovar patoc) to three types of extracellular matrix, including collagen type IV, fibronectin, and laminin which are major components of target organs by ELISA. Both pathogenic and non-pathogenic leptospires bound to all three types of extracellular matrix in the dose-dependent manner and the binding to fibronectin is higher than to collagen and laminin.

In 2008, Saglam and colleagues studied tissue samples from liver, lung, spleen and kidney of 108 ovine aborted fetuses by immunoperoxidase staining technique (antibody against serovar grippotyphosa and hardjo). The results of this study showed that 19 (17%) out of 108 fetuses were positive for the presence of leptospiral antigen. Microscopic studies demonstrated that leptospiral antigens were located in the cytoplasm of macrophages in interalveolar septum of the lung, in the cytoplasm of macrophages in the portal regions and hepatocytes of the liver, in the cytoplasm of epithelial cells of renal pelvis, in the cytoplasm of epithelial cells of cortical and medullar tubules, and macrophages of intertubular region in the kidney. In the spleen, antigens were detected in the cytoplasm of macrophages throughout the parenchymal tissue.

CHAPTER IV
MATERIALS AND METHODS

MATERIALS

1. Microorganisms

The *Leptospira interrogans* serovar pyrogenes isolates (HB001) from a febrile patient who has clinical leptospirosis in Burirum Province. These microorganisms were culture and used in this study. The patient acquired the disease by contact with contaminated water in a ruin pond in Burirum province, October 1999. One to two drops of blood were added to 0.5 ml of protein supplement semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) media containing 100 µg of 5-fluorouracil per ml and incubated at 30˚C. Culture was examined by dark field microscopy every week to confirm the presence of viable leptospires and the absence of contamination. The culture was maintained in screw-cap test tubes containing 0.5 ml of culture from last transfer into a fresh semisolid media every month. Half millimeter of leptospire isolate from stock culture was inoculated into 5 ml of liquid EMJH and incubated at 30˚C. The leptospire isolate grown in the culture for 5 days was centrifuged for 10 min at 5000 rpm. The supernatant was decanted and the leptospire isolated were washed 2 times with phosphate buffer saline (PBS). The isolate was resuspended in PBS and turbidity adjusted to the Mc Farland number 0.5 which corresponded to approximate density of 10^8 leptospires per ml.

2. Animals

Thirty adult female hamsters (*Mesocricetus auratus*) weight between 80-120 grams and age 4-6 weeks were used in this study. They were raised under control-conditioned room, temperature at 25-28˚C with 12 hours light per day throughout the experimental period and were fed on standard diet at the animal room of the Department of Laboratory Animal, Armed Forces Research Institute of Medical Sciences (AFRIMS). They were separated into two groups, the first group was six

control hamsters and the other, experimental groups were *Leptospira interrogans* infected hamsters.

3. Chemicals

3.1 3-aminopropyltriethoxysilane

3.2 Acetone, CH_3COCH_3 (Merck, Germany)

3.3 Deionized water

3.4 Disodium hydrogen orthophosphate, Na_2HPO_4 (SO0337, Scharlau Chemic S.A.)

3.5 Distilled water

3.6 Ethanol, C_2H_6O : 30%, 50%, 70%, 80%, 90%, 95% and absolute (No. 64-17-5, Baker Analyzed®)

3.7 Formaldehyde, HCHO (No. 2209, Ajax Finechem.)

3.8 Glacial acetic acid, CH_3COOH (Baker Analyzed®)

3.9 Glycerol

3.10 Haematoxylin $C_{16}H_{14}O_6$ (HE 0070, Scharlau Chemic S.A.)

3.11 Hydrogen peroxide, H_2O_2 (Merck, Germany)

3.12 Paraplast (BDH Gurr®, VWR international Ltd.)

3.13 Potassium alum, $KA(SO_4)_2$ (AL0746, Scharlau Chemic S.A.)

3.14 Potassium iodate, KI (No. F2G177, Asia pacific specialty chemicals Ltd.)

3.15 Picric acid

3.16 Primary antibody (*Leptospira interrogans* serovar pyrogenes batch#303, Royal tropical Institute KIT Biomedical research, The Netherlands.) (Figure 10)

3.17 Rabbit ABC staining system kit: sc-2018 (Santa Cruiz Biotechnology, Inc.) (Figure 11)

3.17.1 Normal blocking serum 1.0 ml

3.17.2 Biotinylated antibody goat anti-rabbit IgG 250 µg

3.17.3 Avidin 0.5 ml

3.17.4 Biotinylated horseradish peroxidase 0.5 ml

3.17.5 Substrate buffer 10x concentrate 1.0 ml

3.17.6 DAB chromogen 50x concentrate 1.0 ml

3.17.7 Peroxidase substrate 50x concentrate1.0 ml

3.18 Resin solution (Merck, Germany)

3.19 Sodium dihydrogen orthophosphate, NaH_2PO_4 (No. AF502342, Ajax Finechem.)

3.20 Xylene, $C_6H_4(CH_3)_2$ (Baker Analyzed®)

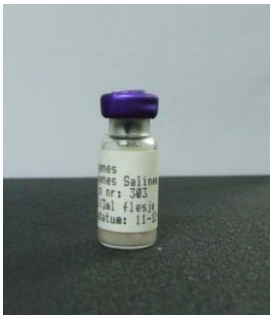


Figure 10 Primary antibody (*Leptospira interrogans* serovar pyrogenes batch#303, Royal tropical Institute KIT Biomedical research, The Netherlands.

Figure 11 Rabbit ABC staining system kit: sc-2018 (Santa Cruiz Biotechnology, Inc.). A: box of product and B: stock solutions in the box (below set) and empty bottles that were used for mixing the working solution (above set).

4. Tools

4.1 Beakers (Schott Duran)

4.2 Coplin jars

4.3 Cover slips (Menzel-Glaser®)

4.4 Cylinders

4.5 Dropper

4.6 Forceps

4.7 Glass bottles

4.8 Glass stirring rod

4.9 Heater (IDAMAG® , REC-G)

4.10 Hot air oven (Memmert)

4.11 Humidity chamber

4.12 Light microscope (Nikon SY 100)

4.13 Light microscope attached digital camera

4.14 Magnetic bar

4.15 Micropipette 5-50 µl and tips (AN 64554, Harikul Calibration center)

4.16 Microscopic glass slides 2.54 x 76.2 mm, 1.2 mm thick (CAT. NO. 7105, HAD)

4.17 Mixing bottle for the preparation working solution in ABC staining system kit

4.18 Molds

4.19 Paraffin dispenser (71010, K.V. Science Co., Ltd.)

4.20 pH meter (Suntex digital model: SP-7)

4.21 Pipettes (Western Germany)

4.22 Rack

4.23 Rotary microtome (Leica RM 2135)

4.24 Rubber Bulb

4.25 Slide boxes

4.26 Staining jars

4.27 Timers

4.28 Vials

4.29 Water Bath (MEDAX, KV-7684)

4.30 Weighing (Precisa XT 320 M)

METHODS

1. Animal inoculation

Six control hamsters injected intraperitoneally with 0.5 ml of PBS served as the control group. The other 24 hamsters were the experimental groups. They were injected intraperitoneally with 0.5 ml of PBS containing 1 x 10^8 leptospires per milliliter.

Three control hamsters were sacrificed on day 2 and the last three on day 5. Each 3 of 24 infected hamsters were sacrificed at 1 hour, 6 hours and on day 1, 2, 3, 4, 5 and 6 after leptospire injection respectively. All animals were killed by first anaesthetizing by CO_2 and then they were put into the CO_2 chamber, after that laid down on plate and the thoracic cavity was opened by subcostal incision. The livers, spleens and lungs of all sacrificed animals were removed

2. Specimen preparation for light microscopy

The organs were washed by many changes of normal saline in order to get rid of the blood. Then they were fixed in Bouin's solution (Appendix A) for 2 days at room temperature. The fixed organs were washed by 50% ethanol for 4-5 minutes followed by 70% ethanol until the yellow color of Bouin's solution was removed. The livers, spleens and lungs were processed for conventional light microscopy (Appendix B). After processing, the tissue blocks were cut at a thickness of 2 µm with a rotary microtome and mounted on coated glass slides with 3-aminopropyltriethoxysilane (Appendix C). Then these sections on the coated slides were incubated in the oven at 56-60˚C for 30 minutes.

3. Indirect immunoperoxidase staining technique

The sequence of this staining technique followed the rabbit ABC staining system kit protocol. These sections were deparafinized before being stained with indirect immunoperoxidase technique by using rabbit primary antibody to *Leptospira interrogans* serovar pyrogenes and rabbit ABC staining system kit. Then the sections were mounted with resin solution (Appendix D).

Principle of immunoperoxidase staining technique

The immunohistochemistry refers to the process of localizing the protein in the cell of tissue section exploiting the principle of antibodies binding specifically to antigens (Ramos-Vara, 2005).

The indirect method used primary antibody against the antigen being probed and the labeled secondary antibody against the primary antibody. This method involved an unlabelled primary antibody (first layer) which reacted with tissue antigen and a labeled secondary antibody (secondary layer) which reacted with the primary antibody. The secondary antibody was against the IgG of the primary antibody. This method was more sensitive due to signal amplication. The second layer antibody was conjugated with the biotin on Fc fragment (called biotinylated secondary antibody). The avidin-biotin peroxidase complex was applied. Avidin was a large glycoprotein extracted from the egg white that had four binding sites for low molecular weight vitamin, called biotin (Ramos-Vara, 2005). The biotinylated secondary antibody performed with avidin-biotin peroxidase complex. The peroxidase was an enzyme and the horseradish peroxidase was commonly used. The peroxidase had iron (containing heme group or hematin) as its active site and in solution was colored brown. The hematin of the peroxidase first formed a complex with hydrogen peroxide (H_2O_2) and then caused it to decompose resulting in water and oxygen atom (Naish, 1989). The final step, the peroxidase was visualized with the usual substrate by activity of the peroxidase. The oxygen (O_2) was released from H_2O_2 and then oxidized 3, 3´ Diaminobenzidine tetrahydrochloride (DAB) to brown coloration end product (Figure 12) (Wordinger *et al*., 1983).

Regarding to the experimental negative control, the primary antibody was omitted however, it was used in the staining procedure of the uninfected control groups.

4. Examination of the section

All the stained sections of each organ mentioned from both control groups and the infected groups were examined carefully under the light microscope. The leptospiral antigens that appeared as the golden-brown precipitation in each organ tissue of the infected groups were recorded and photographed by the attached digital camera on the light microscope.

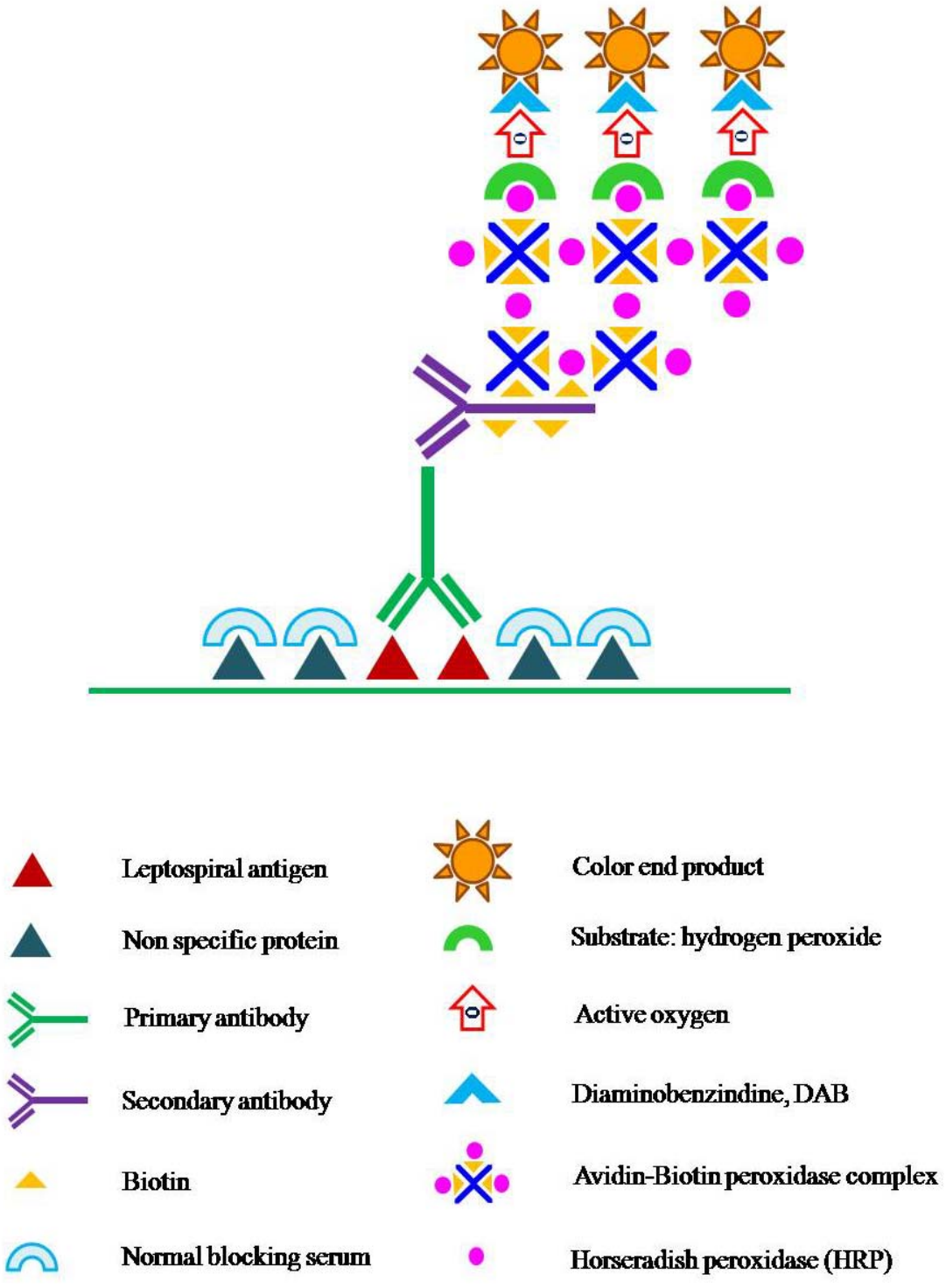


Figure 12 Diagram illustrating indirect immunoperoxidase stain by Avidin-Biotin complex (Adapted from Naish, 1989).

CHAPTER V
RESULTS

Liver (Table 3)

The liver tissue of the control groups and the experimental negative staining control of the infected group illustrated no any golden brown coloration in the sections, including the portal triad, hepatic parenchyma and the central vein. These sections are expressed as the blue stain of the Carazzi's haematoxylin solution (Figure 13)

In the liver tissue of different experimental groups, such as the portal triad, hepatic parenchyma and the central vein displayed the positive golden brown staining of various intensities.

Portal triad (Figure 14)

The epithelial lining cells of the bile duct demonstrated the mild golden brown coloration in all infected groups from one hour to six days post infection (Figure 14).

The endothelial cells and the smooth muscle cells of the hepatic artery showed the mild golden brown coloration in all infected groups (Figure 14). In addition, the various intensities of the positive stained content in this lumen was observed in all groups post infection from first hour to six days except the group of one day. The mildly stained content was observed in the group of one hour (Figure 14A), six hours (Figure 14B), four days (Figure 14F) and five days (Figure 14G). The content appeared intensive positive stain in the group of two days (Figure 14D), three days (Figure 14E) and six days (Figure 14H).

The portal vein appeared mildly stained cytoplasm of the endothelial cells in all infected groups from one hour to six days post infection (Figure 14). Moreover, the intensively stained content in the lumens were observed in the groups of one hour to two days (Figure 14A-D) and six days (Figure 14H). Besides, the moderately stained

luminal content could be found in the groups of three days to five days (Figure 14E -G).

Hepatic parenchyma (Figure 15)

The golden brown stained cytoplasm of the hepatocyte was observed in all experimental groups. The cytoplasm and boundary of the hepatocytes appeared the leptospiral antigen depositing nearby the portal area in the early groups of infection and in the late groups. These antigens were found in the distance between the portal area and central vein. Some of hepatocytes in the one hour group after infection showed the mildly stained cytoplasm and intensive golden brown coloration at the border of one cell (Figure 15A). The intensively stained cytoplasm of the hepatocytes was appeared in the group of six hours (Figure 15B). However, the positive stained hepatocytes in one and six hour experimental groups were rarely observed. The hepatocytes demonstrated the moderately stained cytoplasm around the portal triad in the one day group post infection. However, the mild golden brown stain could be found in the cytoplasm of the hepatocyte in this group (Figure 15C). Moreover, the mild golden brown coloration was mostly observed in the group of two days to six days, (Figure 15D-G). Most hepatocytes lose contact with each other making the discohesion of the hepatic cord in the group of five and six days. These hepatocytes showed the intensive golden brown coloration along the boundary of the cells (Figure 15G and H).

The hepatic sinusoid displayed the mildly stained cytoplasm of the endothelial cells that lining the sinusoid in the group of one hour to two days (Figure 15A-D), intensively in the group of three days (Figure 15E) and moderately in the groups of four to six days (Figure 15F-H). The content in the sinusoidal lumen showed intensive stain in the groups of three days (Figure 15E) and moderate stain in the groups of four to six days (Figure 15F-G).

The inflammatory cells mainly neutrophils were found in the sinusoidal lumen and expressed the positive stain of different intensity in the experimental groups from first day to six days. For instance, the intensively stained cytoplasm appeared in the neutrophil of the two (Figure 15F) and four days, moderately in the group of six days (Figure 15H) and mildly in the groups of one day, three days and five days.

Additionally, the some Kupffer cells displayed the intensively stained cytoplasm in the five days group (Figure 15G).

Central vein (Figure 16)

The endothelial lining and the content of the central vein revealed the positive golden brown stain in all infected groups. However, the pattern of the positive stained area was different. The intensively stained cytoplasm of the endothelium was observed in the group of one hour (Figure 16A), five days (Figure 16G) and six days (Figure 16H), moderately in the groups of two days (Figure 16D) and three days (Figure 16E) as well as mildly in the groups of six hours (Figure 16B), one day (Figure 16C) and four days (Figure 16F). The luminal content which was mainly the red blood cells also showed the positive stain of various intensities in all experimental groups. The intensive stain was expressed in the groups of one hour (Figure 16A), five days (Figure 16G) and six days (Figure 16H), moderate in the groups of six hours to three days (Figure 16B-E) and mild in the group of four days (Figure 16F).

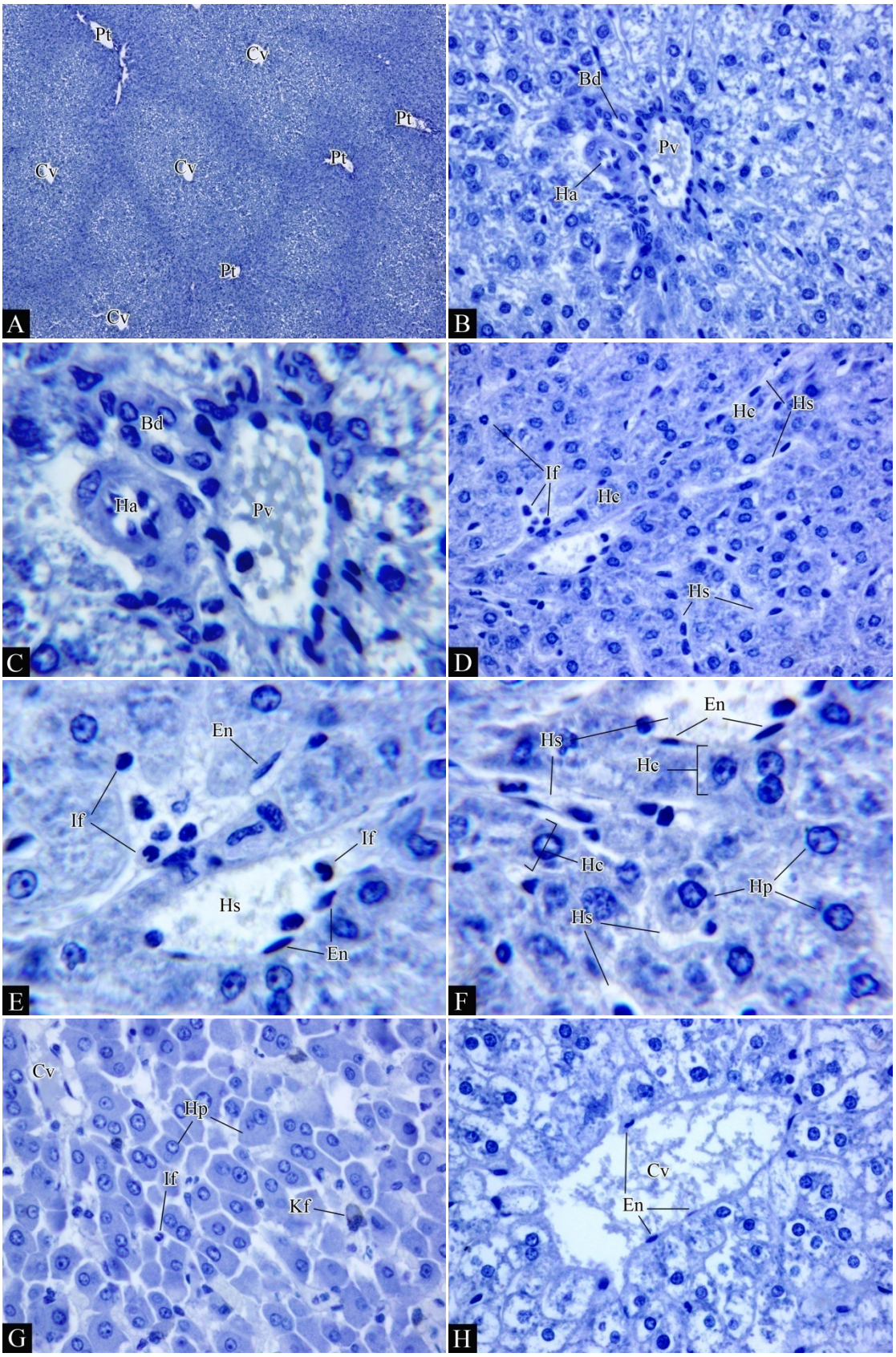
Table 3 The distribution of leptospiral antigen in the liver tissue.

Type of cell	Infection time periods							
	1 hr.	**6 hrs.**	**1 day**	**2 days**	**3 days**	**4 days**	**5 days**	**6 days**
Portal triad								
● Bile duct								
- Epithelium	+	+	+	+	+	+	+	+
● Hepatic artery								
- Endothelial cell	+	+	+	+	+	+	+	+
- Smooth muscle cell	+	+	+	+	+	+	+	+
- Content	+	+	-	+++	+++	+	+	+++
● Portal vein								
- Endothelial cells	+	+	+	+	+	+	+	+
- Content	+++	+++	+++	+++	++	++	++	+++
Hepatic parenchyma								
● Hepatocyte	+	+++	++	+	+	+	+	+
● Hepatic sinusoid								
- Endothelial cell	+	+	+	+	+++	++	++	++
- Content	-	-	-	-	+++	++	++	++
- Inflammatory cell	-	-	+	+++	+	+++	+	++
Central vein								
- Endothelial cell	+++	+	+	++	++	+	+++	+++
- Content	+++	++	++	++	++	+	+++	+++

(-) negative stain, (+) mild positive stain, (++) moderate positive stain and (+++) intensive positive stain

Figure 13 Photomicrographs illustrating the experimental negative control of the liver in the group of one hour (A), one day (B and C), four days (D-F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes. The pictures display various structures of the liver tissue, including the hepatic parenchyma (A) (Obj 10x), the portal triad (B and C) (Obj 40x and 100x respectively), the hepatic sinusoid (Hs) and hepatic cord (Hc) (D-F) (D, Obj 40x; E and F, Obj 100x), discohesion of hepatic cord (G) (Obj 40x) and the central vein (Cv) (H) (Obj 40x). No any positive golden brown stained area is seen in the sections.

Abbreviation: Bd, Interlobular branch of bile duct; En, Endothelial cell; Ha, Interlobular branch of hepatic artery; If, Inflammatory cell; Kf, Kupffer cell; Pv, Interlobular branch of portal vein.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Hp
Hs
F
Cv
Hp
If
Kf
G
Cv
En
H

Figure 13

Figure 14 Photomicrographs of the infected liver illustrating the portal triad, including interlobular branch of bile duct (Bd), interlobular branch of hepatic artery (Ha) and interlobular branch of portal vein (Pv) in the group of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes.

A) The mild golden brown coloration is demostrated at the luminal surface of the epithelial lining cells of the bile duct (Bd), in the endothelium of the hepatic artery (Ha) and portal vein (Pv) (arrow head). The contents in the lumen of hepatic artery and portal vein display mild to intensive golden brown stain. (Obj 100x)

B-H) The endothelial cells (En) and smooth muscle cells (Sm) of the hepatic artery, portal vein and epithelial cells of the bile duct appear mildly stained cytoplasm. The contents in the lumen of blood vessels show various positive stain. The lumen of the portal vein reveals intensive stain (B-D and H) and moderate stain (E-G) while the lumen of the hepatic artery displays intensive stain (D-E and H) and mild stain (B and F-G). (B and D, Obj 100x; C and G, Obj 40x; E, Obj 20x and inset 40x; F, Obj 40x and inset 100x)

Abbreviation: If, Inflammatory cell; Hs, Hepatic sinusoid.

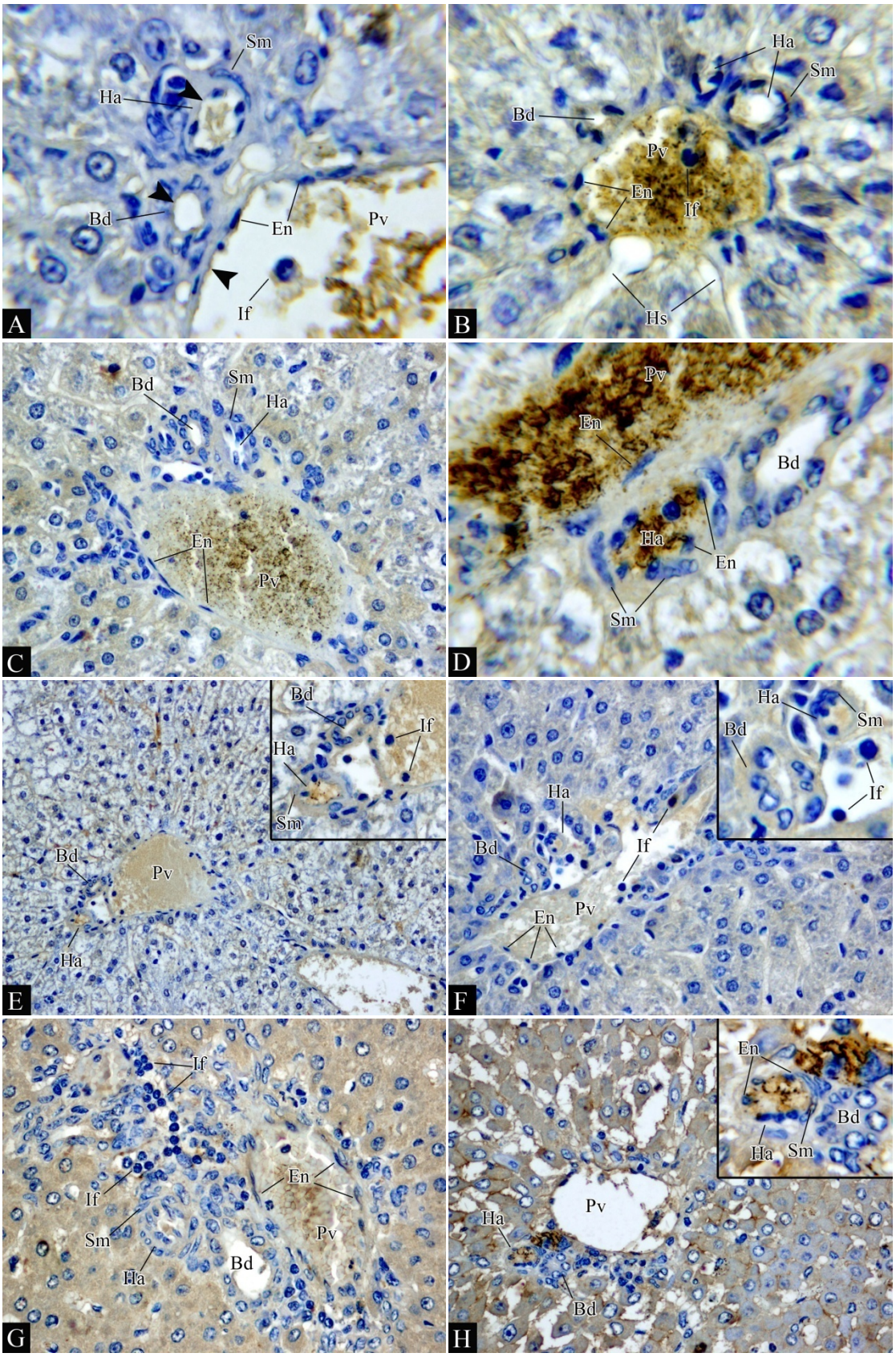
A
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Figure 14

Figure 15 Photomicrographs of the infected liver illustrating the hepatocyte, hepatic cord and hepatic sinusoid in the group of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes.

A) The intensive golden brown coloration is demonstrated at the border of one hepatocyte (He) (arrow head). (Obj 40x and inset 100x)

B) Some hepatocytes appear the intensive stained cytoplasm (arrow head). (Obj 40x)

C) The mild (red arrow head) and moderate (black arrow head) golden brown stain is revealed in the cytoplasm of the hepatocytes. (Obj 20x)

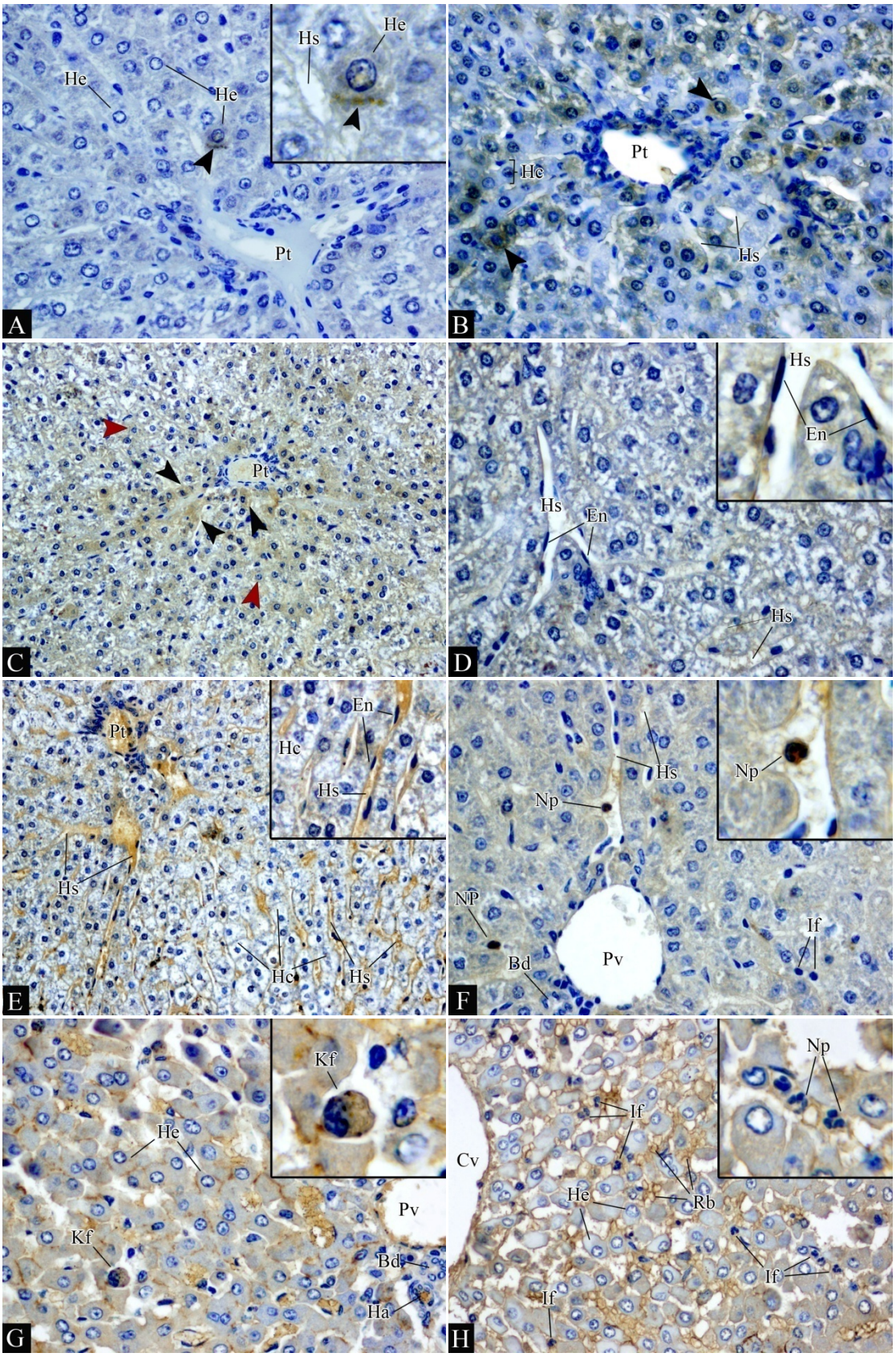
D The hepatocytes reveal mild stain and the endothelial lining cells of the hepatic sinusoid (Hs) show the mild stain. (Obj 40x and inset 100x)

E) The intensive golden brown stain is demonstrated in the cytoplasm of the endothelial cells lining the hepatic sinusoid and in the content of the lumen. The hepatocytes show mild stain. (Obj 20x and inset 40x)

F) The intensive stained cytoplasm of the neutrophils (Np) is observed in the hepatic sinusoid. The wall and the lumen of the hepatic sinusoid appear moderate golden brown coloration. Additionally, the cytoplasms of the hepatocytes display mildly positive stain. (Obj 40x and inset 100x)

G-H) The hepatocytes show the intensive golden brown coloration along the boundary of the cells and mild stain in the cytoplasm. Most of hepatocytes lose contact with each other making the discohesion of the hepatic cords. In addition, the hepatic sinusoid is congested with red blood cells, moderately stained inflammatory cells (If) and intensively stained kupffer cell (Kf). (Obj 40x and inset 100x)

Abbreviation: Bd, Interlobular branch of bile duct; Cv, Central vein; Ha, Interlobular branch of hepatic artery; Hc, Hepatic cord; Pv, Interlobular branch of portal vein; Pt, Portal triad.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Cv
He
Rb
If
If

Figure 15

Figure 16 Photomicrographs of the infected liver illustrating the central vein in the group of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes.

A) The endothelial cells (En) appear intensively stained cytoplasm. The contents in the lumen also reveal intensive golden brown coloration. (Obj 40x and inset 100x)

B-C) The mildly stained cytoplasm is displayed in the endothelial cells and the mild (red arrow head) and moderate (black arrow head) stain are demonstrated in the lumen. (Obj 40x and inset 100x)

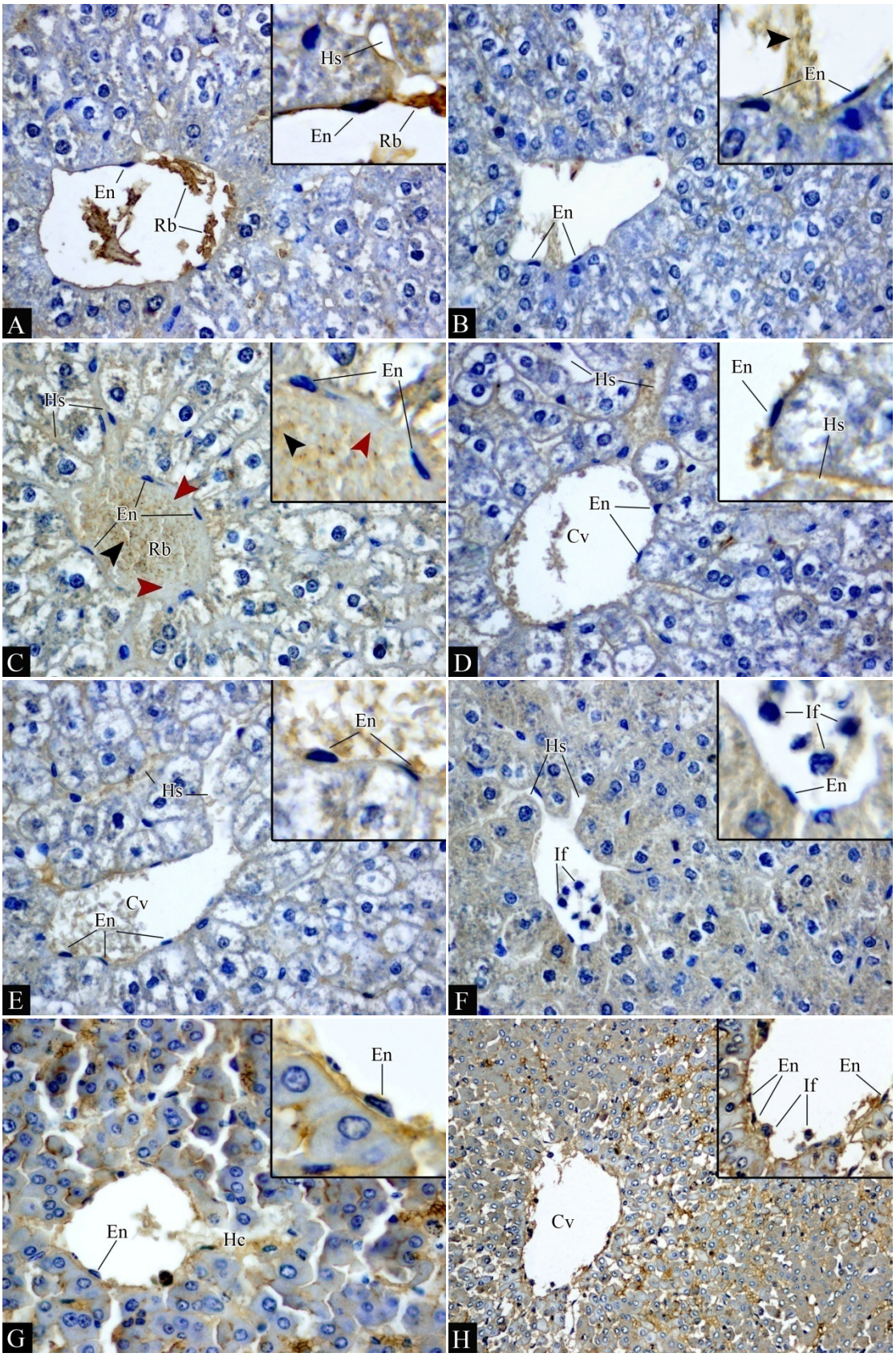
D) The cytoplasm of endothelial cells of the central vein and hepatic sinusoid as well as the content in the lumen show moderate stain. (Obj 40x and inset 100x)

E) The cytoplasm of the endothelial cells and contents in the lumen of the central vein display moderate positive stain. (Obj 40x and inset 100x)

F) The mild golden brown stain is illustrated in the cytoplasm of the endothelial cells and inflammatory cells locating inside the lumen. (Obj 40x and inset 100x)

G-H) The cytoplasm of the endothelial cells lining the central vein and inflammatory cells locating inside the lumen appear intensive golden brown coloration. (G, Obj 40x; H, Obj 20x and inset 40x)

Abbreviation: Rb, Red blood cell.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En
If
En
Cv

Figure 16

Spleen (Table 4)

The splenic tissue of the control groups and the experimental negative staining control of the infected groups expressed no any golden brown coloration in the sections, including the capsule, trabeculae, central artery, white pulp and red pulp. The sections are displayed as the blue stain of the Carazzi's haematoxylin solution (Figure 17).

Capsule and trabeculae (Figure 18)

The capsule of the spleen demonstrated various intensities of positive golden brown staining in different experimental groups. The moderate golden brown coloration of the capsule was observed in the groups of one hour (Figure 18A) and five days (Figure 18G) and the mild positive stain was found in the groups of six hours (Figure 18B) and one day (Figure 18C) as well as intensive stain in the groups of two days (Figure 18D), three days (Figure 18E), four days (Figure 18F) and six days (Figure 18H). Besides, the trabeculae that emanated from the capsule into the splenic parenchyma showed the positive stained in all groups post infection. The mild positive stain was presented in the group of one hour to three days and five days (Figure 18A-E and G) while the intensive and moderate stain was illustrated in the group of the four days (Figure 18F) and six days (Figure 18H) respectively.

Central artery (Figure 19)

The central artery in the white pulp of different experimental groups illustrated the positive stain of different intensities in all infected groups from the first hour to six days post infection. The moderate stained cytoplasm of the endothelial cells and the smooth muscle cells were presented in the groups of one hour to two days post infection (Figure 19A-D). The cytoplasm of the endothelial cells and the smooth muscle cells in the group of three and four days appeared mild and moderate stain respectively (Figure 19E-F). Moreover, the mildly stained cytoplasm of the endothelial cells was observed in the five and six days groups, whereas the cytoplasm of the smooth muscle in the group of five days displayed intensive stain and six days showed mild stain (Figure 19G-H).

Plasma cell (Figure 20)

The plasma cells in the white pulp of the one hour after infection displayed negatively stained cytoplasm (Figure 20A). The cytoplasm of the plasma cells in the group of six hours to six days appeared mild golden brown stain (Figure 20B-H). However, in the group of five and six days, intensively and moderately stained cytoplasms of the plasma cells were also found respectively (Figure 20G and H). The plasma cells that displayed positive stain were rarely seen in the group of six hours to two days. Then, the number of positively stained plasma cells increased from the groups of three days to six days.

Red pulp (Figure 21)

The structures of the red pulp illustrated the positive golden brown coloration of various intensities from the groups of first hour to six days post infection. The structures in the group of first hour after infection, including the venous sinus, macrophages, neutrophils and red blood cells appeared mild positive stain but were rarely seen. In this group, the aggregation of the intensively stained fine filamentous form was observed in the red pulp (Figure 21A). The endothelia of venous sinus displayed the mild golden brown coloration in all group post infection (Figure 21D and F-G) and the blood elements in the lumen showed various intensities from mild to moderate positive stained.

The macrophages presented the mildly stained cytoplasm in the groups of one hour to five days. In the group of six days, the cytoplasm of the macrophage showed moderate positive stain (Figure 21H). However, the mildly stained cytoplasm of this cell could be found in this group. The number of the macrophages became increasing in the later period. The inflammatory cells mainly neutrophils were observed with different intensities of positive stain in this pulp (Table 4). The neutrophils were found prominently in the red pulp nearby the marginal zone. This phenomenon was demonstrated in the one day to six days after infection (Figure 21C). The red pulp was occupied with red blood cells. These cells showed different intensities of positive stain, mainly mild golden brown stain in the one hour group (Figure 21A) and moderate positive stain in the six hours to six days groups post infection (Figure 21B-H) (Table 4).

Table 4 The distribution of leptospiral antigen in the splenic tissue.

Type of cell	Infection time periods							
	1 hr.	**6 hrs.**	**1 day**	**2 days**	**3 days**	**4 days**	**5 days**	**6 days**
Capsule	++	+	+	+++	+++	+++	++	+++
Trabeculae	+	+	+	+	+	+++	+	++
Central artery								
● Endothelial cell	++	++	++	++	+	+	+	+
● Smooth muscle cell	++	++	++	++	++	++	+++	+
White pulp								
● Plasma cell	-	+	+	+	+	+	+ and +++	+ and ++
Red Pulp								
● Venous sinus	+	+	+	+	+	+	+	+
● Macrophage	+	+	+	+	+	+	+	++
● Neutrophil	+	++	+++	++	+	+++	+++	+++
● Red blood cell	+	++	++	++	++	++	++	++

(-) negative stain, (+) mild positive stain, (++) moderate positive stain and (+++) intensive positive stain

Figure 17 Photomicrographs illustrating the experimental negative control of the spleen in the group of six hours (A), four days (B-E) and five days (F) post infected hamsters with *Leptospira interrogans* serovar pyrogenes. The pictures display various structures of the spleen tissue, including the capsule (Cs) and trabeculae (Ta) in A (Obj 40x); the low magnification of the splenic parenchyma (B) (Obj 10x) and the high magnifications of central artery (C) (Obj 40x); white pulp (D) (Obj 40x and inset 100x); venous sinus (Vs) in the red pulp (E) (Obj 40x) and cells in the red pulp (F) (Obj 40x and inset 100x). No any positive golden brown stained area is seen in the sections.

Abbreviation: Ce, Central artery; En, Endothelial cell; If, Inflammatory cell; Mc, Macrophage; Pm, Plasma cell; Rb, Red blood cell; Rp, Red pulp; Sm, smooth muscle; Wp, White pulp.

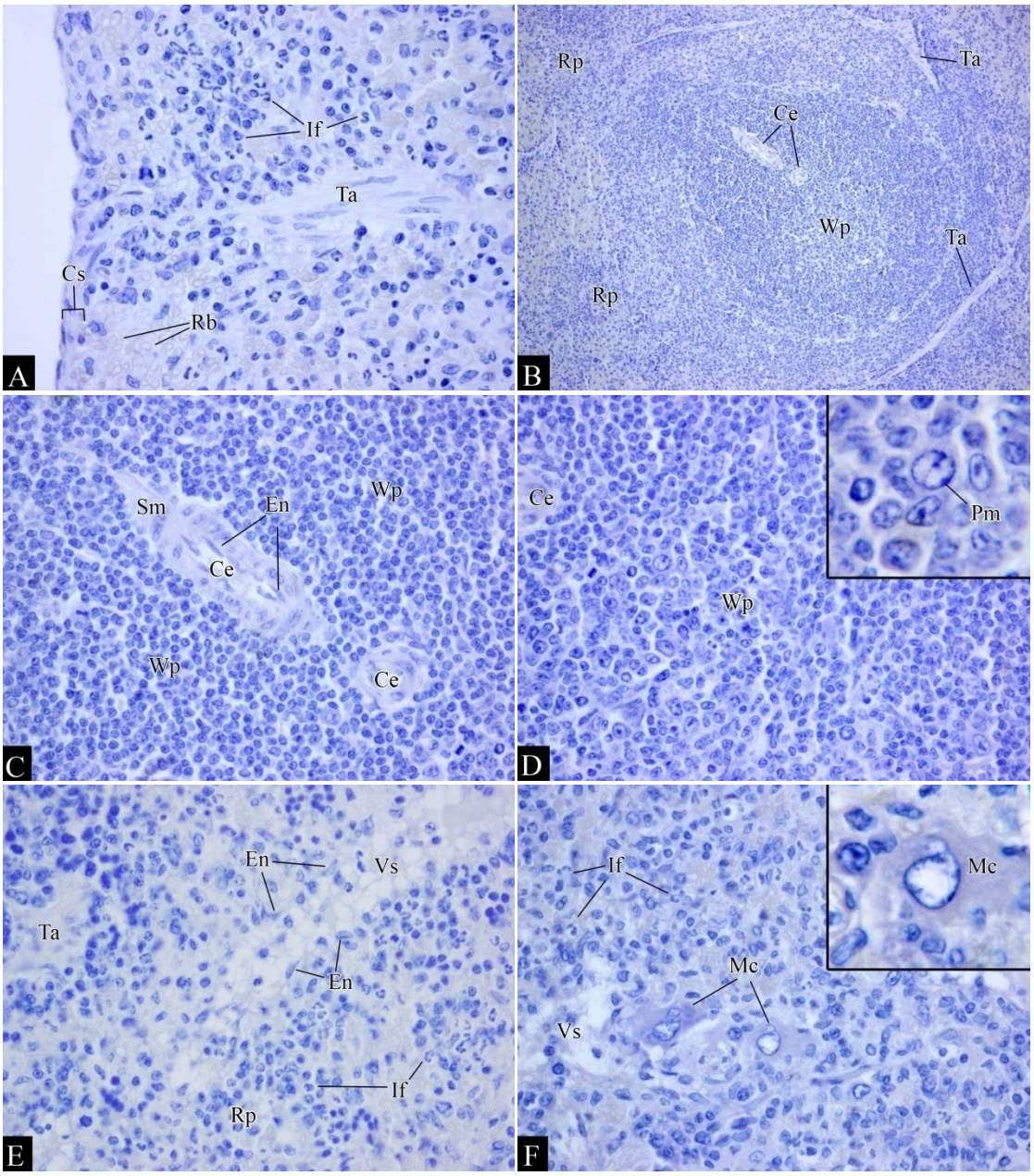

A
If
Ta
Cs
Rb
B
Rp
Ta
Ce
Wp
Ta
Rp
C
Sm
En
Wp
Ce
Wp
Ce
D
Ce
Pm
Wp
E
En
Vs
Ta
En
If
Rp
F
If
Mc
Mc
Vs

Figure 17

Figure 18 Photomicrographs of the spleen illustrating the capsule and trabeculae in the group of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes. (Obj 40x).

A) The capsule (Cs) of the spleen appears positive moderate golden brown coloration and the trabeculae (Ta) shows mild stain.

B-C) The capsule and trabeculae of the infected spleen illustrate mild positive stain.

D-E) The intensive golden brown stain is demonstrated in the capsule while the trabeculae appears mild positive stain.

F) The capsule and trabeculae reveals intensive positive stain.

G) The capsule appears moderate golden brown coloration and trabeculae shows mild positive stain.

H) The intensive stain appears in the capsule whereas the trabeculae show moderate golden brown stain.

Abbreviation: En, Endothelial cells; If, Inflammatory cell; Rb, Red blood cell; Vs, Venous sinus.

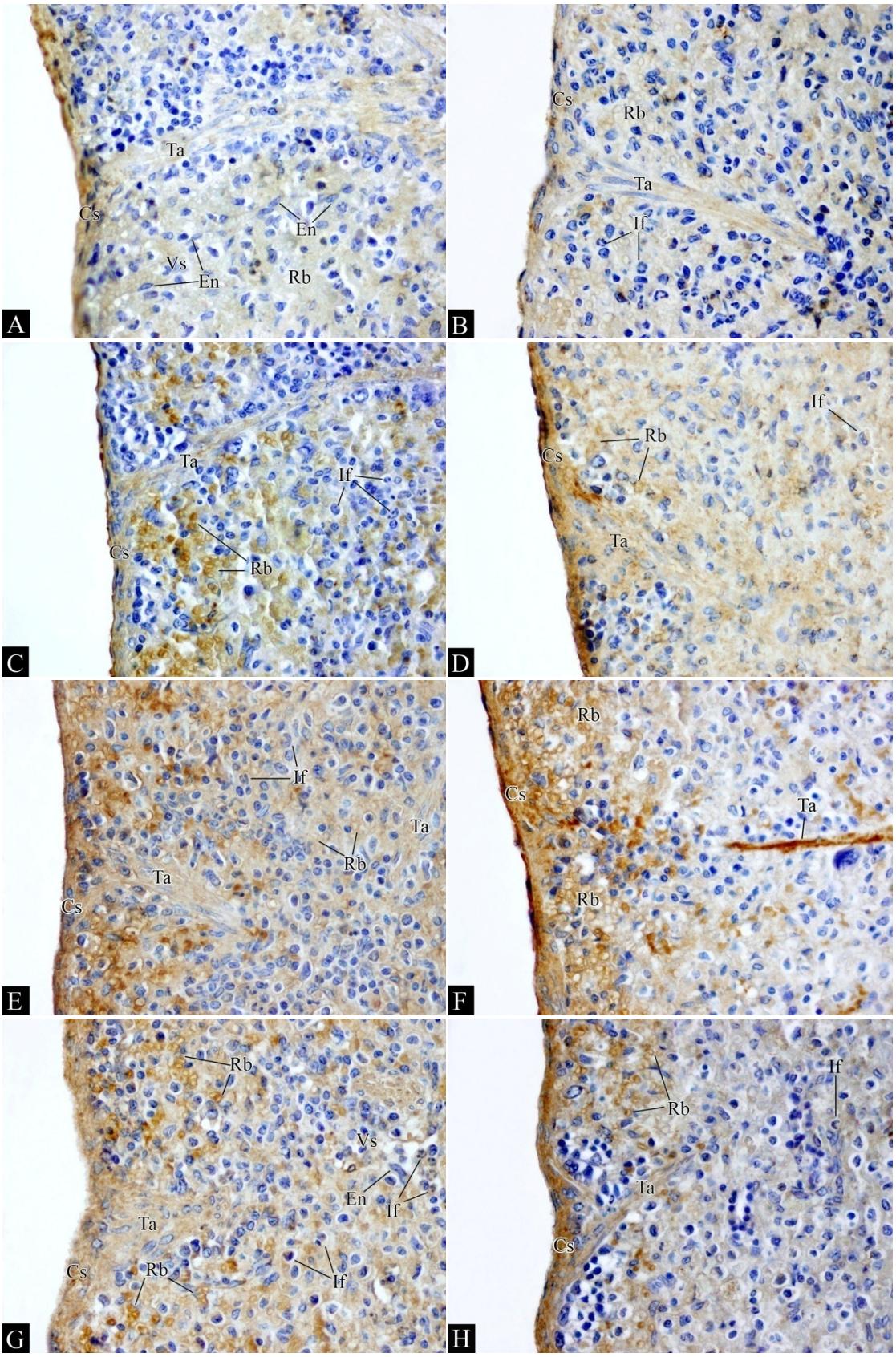

A
Ta
Cs
En
Vs
En
Rb
B
Cs
Rb
Ta
If
C
Ta
If
Cs
Rb
D
If
Rb
Cs
Ta
E
If
Ta
Rb
Ta
Cs
F
Rb
Cs
Ta
Rb
G
Rb
Vs
En
If
Ta
Cs
Rb
If
H
If
Rb
Ta
Cs

Figure 18

Figure 19 Photomicrographs of the infected spleen demonstrating the central artery in the white pulp in the group of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes.

A-D) The infected spleen showing the central artery appear the moderately stained cytoplasm of the endothelial cells (En) and the smooth muscle cells. (A and C, Obj 40x and inset 100x; B and D, Obj 100x)

E-F) The endothelial cells of the central artery appear the mildly stained cytoplasm and the smooth muscle cells shows the moderately stained cytoplasm. (Obj 100x)

G) The endothelial cells and the smooth muscle cells of the central artery appear the mildly and intensively stained cytoplasm respectively. (Obj 100x)

H) The central artery appears mild golden brown coloration in the cytoplasm of the endothelial cell and the smooth muscle cells. (Obj 100x)

Abbreviation: Ce, Central artery; Rp, Red pulp; Wp, White pulp.

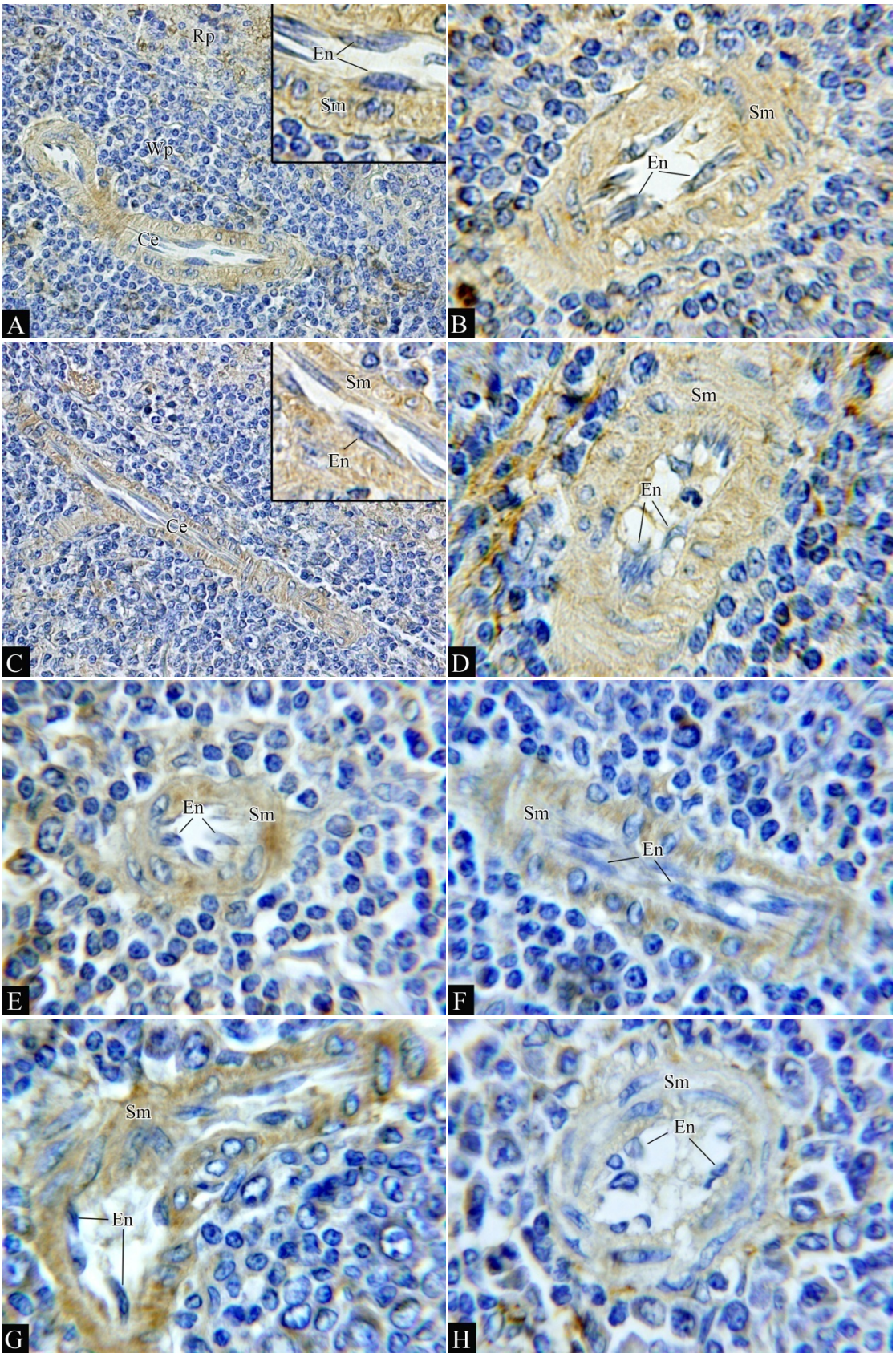


Figure 19

Figure 20 Photomicrographs of the infected spleen illustrating the plasma cells in the white pulp in the group of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes. (Obj 40x and inset 100x)

A) The plasma cells appear negative golden brown coloration (arrow head).

B) The mild stain is displayed in the cytoplasm of the plasma cells (arrow head) while red blood cells (Rb) in the pulp appear intensive positive stain.

C-F) The plasma cells reveal the mildly stained cytoplasm (arrow head).

G) The white pulp demonstrates both mildly (black arrow head) and intensively (red arrow head) stained cytoplasm of the plasma cells.

H) The plasma cells perform the mildly (black arrow head) and moderately (red arrow head) stained cytoplasm. (Obj 40x and inset 100x)

Abbreviation: Ce, Central artery; Rp, Red pulp; Wp, White pulp.

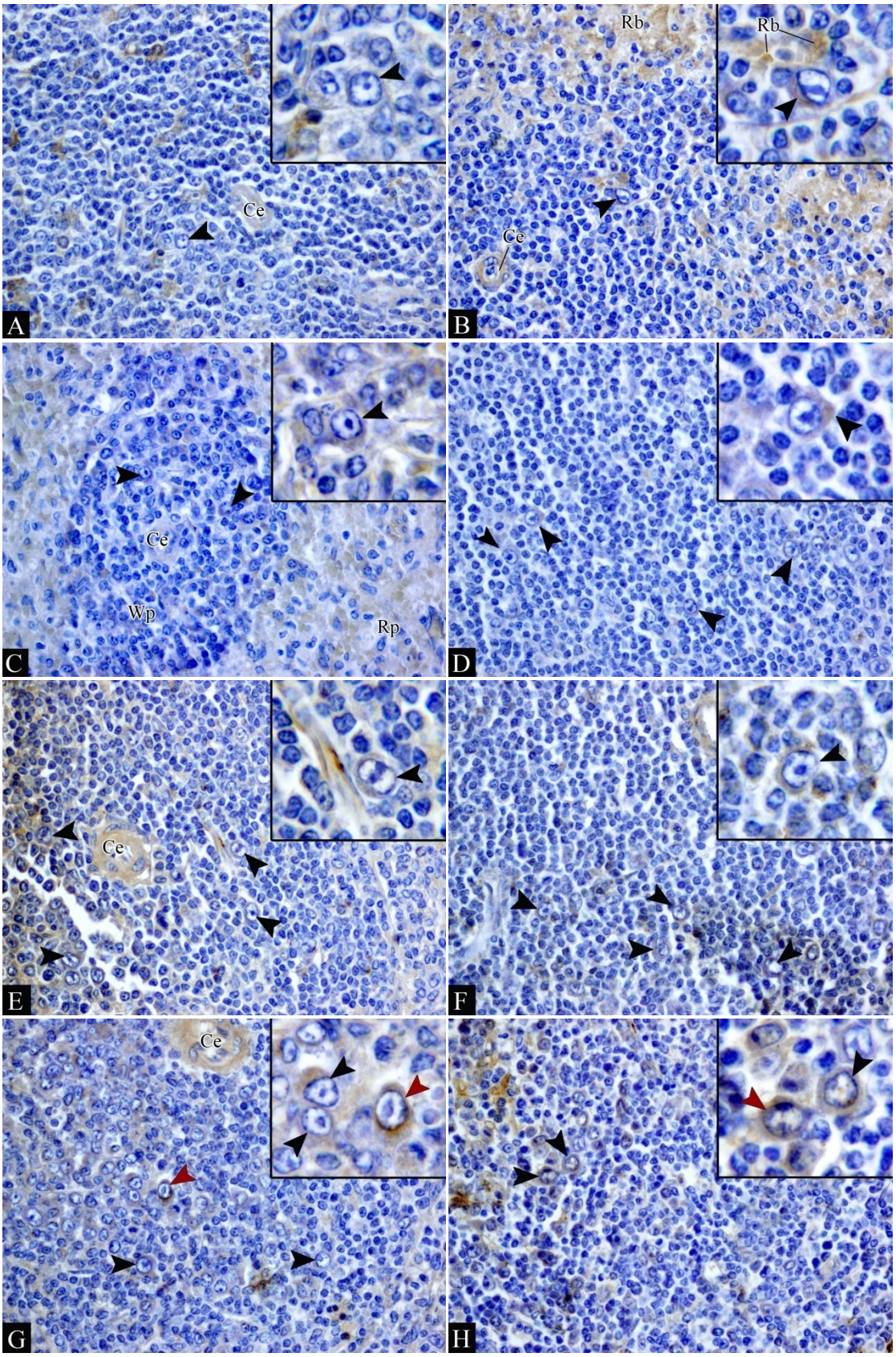


Figure 20

Figure 21 Photomicrographs of the infected spleen demonstrating the red pulp in the group of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes.

A) The red pulp (Rp) appears the aggregation of the intensively stained fine filamentous forms (arrow head). (Obj 20x and inset 100x)

B) The red blood cells (Rb) reveal the moderate golden brown coloration. Some neutrophils (Np) appear moderate stain. (Obj 40x and inset 100x)

C) The red blood cells appear the moderate golden brown coloration. A few neutrophils in the red pulp and in the marginal zone (Mg) of white pulp show the intensively stained cytoplasm. (Obj 40x and inset 100x)

D) The endothelial cells (En) of the venous sinus (Vs) display mild to moderate stained cytoplasm. (Obj 40x and inset 100x)

E) The red blood cells appear mild to moderate golden brown coloration and some neutrophils show mildly stained cytoplasm. (Obj 40x and inset 100x)

F) The endothelial cells of the venous sinus appear mild golden brown coloration and the lumen is congested with red blood cells and neutrophils. Some neutrophils appear intensive stain. (Obj 40x and inset 100x)

G) The venous sinus shows the mild stain and the lumen is congested with mildly stained red blood cells and inflammatory cells. (Obj 20x and inset 40x)

H) The red blood cells appear moderate to intensive stain and the macrophage (Mc) shows moderately stained cytoplasm. (Obj 40x and inset 100x)

Abbreviation: Ta, Trabecular; Wp, white pulp.

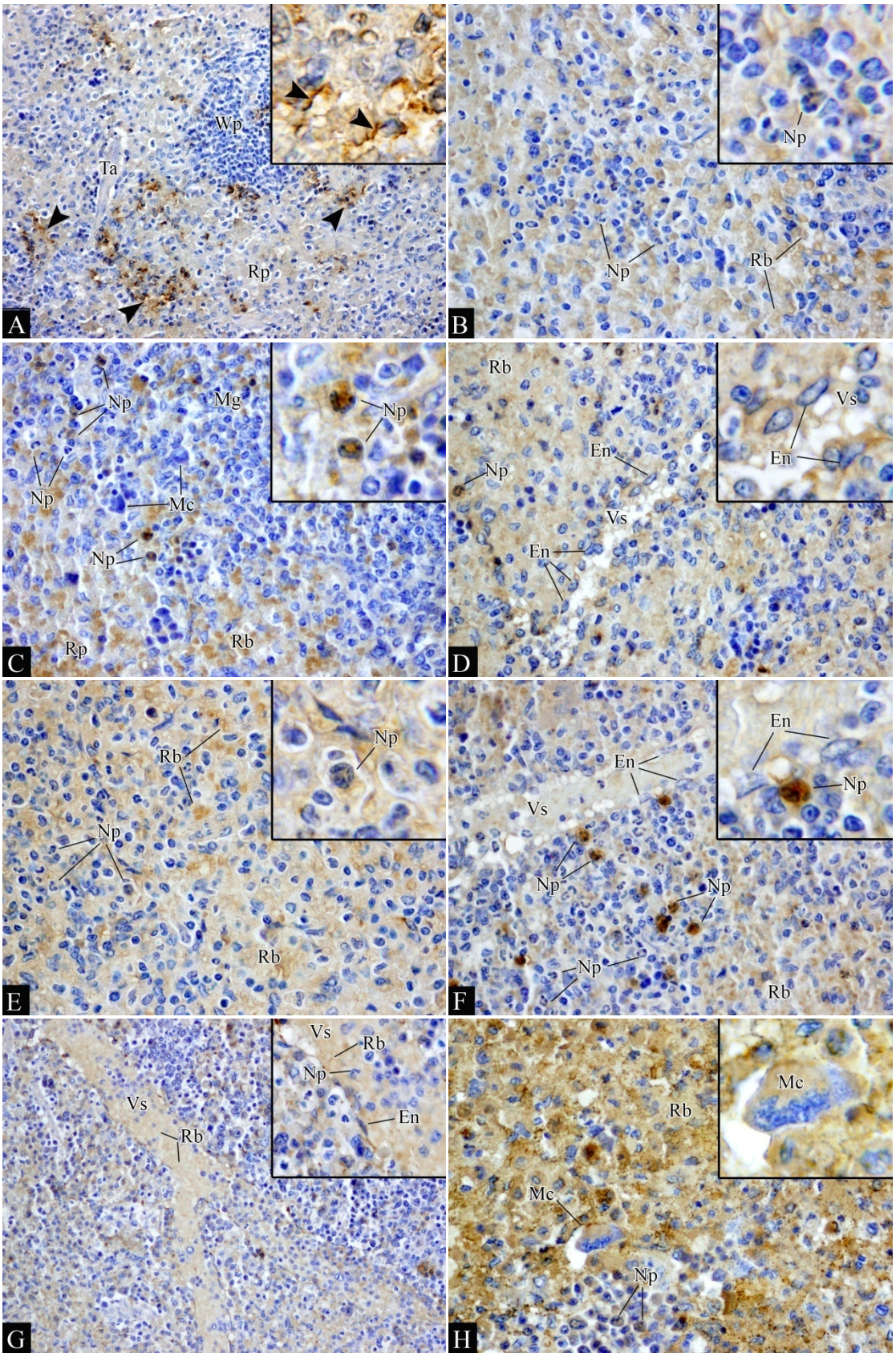

A
Wp
Ta
Rp
B
Np
Np
Rb
C
Np
Mg
Np
Np
Mc
Np
Rp
Rb
D
Rb
Vs
En
Np
En
Vs
En
E
Rb
Np
Np
Rb
F
En
En
Np
Vs
Np
Np
Np
Rb
G
Vs
Rb
Np
En
Vs
Rb
H
Mc
Rb
Mc
Np

Figure 21

Lung (Table 5)

The tissue of the control groups and the experimental negative control of the infected groups, including the bronchus, bronchiole, terminal bronchiole, respiratory bronchiole, alveolar duct, alveolar sac, alveolus, pulmonary arteriole, small vein and venule showed no any golden brown stain in the sections. These tissue sections displayed the blue color stained of the Carazzi's haematoxylin solution (Figure 22).

The leptospiral antigens in the lung tissue of the infected hamsters had been characterized by indirect immunoperoxidase staining technique. The positive golden brown coloration should display the area where the antigens were depositing.

Bronchus (Figure 23)

The bronchus demonstrated the positive stained in all infected groups with various intensities from the groups of first hour to six days. The epithelial cells of the bronchus showed the mildly stained cytoplasm (Figure 23). The lamina propria layer demonstrated positive golden brown stain in various intensities, regarding to mild (Figure 23D), moderate (Figure 23B and H) and intensive (Figure 23D) and the venule in this layer illustrated intensive positive stain (Figure 23C). The lumen was congested with red blood cells and neutrophils which appeared as mild and intensive positive stained respectively (Figure 23C). Moreover, some bronchus was occupied by blood elements consisting of red blood cells and inflammatory cells. They appeared positive stained in various intensities from mild to intensive (Figure 23A, D-F and H). The intensive golden brown coloration was performed in the cytoplasm of the smooth muscle cells in all experimental groups (Figure 23A – C and G). In addition, the connective tissue in the lung stroma expressed the intensive stain (Figure 23A-B) and the perichondrium demonstrated the moderate to intensive stain (Figure 23A-D and F).

Bronchiole (Figure 24)

The cytoplasm of the epithelial cells of the bronchioles, terminal bronchioles and respiratory bronchioles displayed the golden brown coloration with various intensities in all of the infected groups. The cytoplasm of the epithelia appeared intensive in the groups of first hour (Figure 24A), six hours (Figure 24B) and five days (Figure 24G);

moderate in the groups of three (Figure 24E), four (Figure 24F) and six days (Figure 24H); mild in the groups of one (Figure 24C) and two (Figure 24D) days.

The smooth muscle cells of bronchioles showed the positive mild and intensive coloration. The intensive stain was observed in the groups of one (Figure 24A) and six hours (Figure 24B) while the mild stain was revealed in the groups of one day to six days (Figure 24C-H). In addition, the connective tissue showed intensive coloration in the groups of one hour to two days (Figure 24A-D), moderate in the groups of five days (Figure 24G) and mild in three (Figure 24E), four (Figure 24F) and six (Figure 24H) days.

Alveolus (Figure 25)

The various intensities of golden brown coloration were performed in alveolar epithelium of all groups after infection from one hour to six days. The moderately stained cytoplasm of the alveolar epithelium was displayed in first and six hours after infection (Figure 25A-B). In addition, the intensively stained fine filamentous structures attaching at the apical border of some alveolar epithelial cells was displayed in first hour after infection (Figure 25A). Later, most of alveolar epithelial cells of one and two days groups after infection illustrated the mildly stained cytoplasm (Figure 25C and D), even so the moderate and intensive positive stain could be found in some areas. Then, the moderately positive stained cytoplasm of the alveolar epithelium was revealed again in the groups of three to four days after infection (Figure 25E-F). Finally, the alveolar epithelial cells became mildly stained cytoplasm again in the five and six days group after infection (Figure 25G-H). The neutrophils that deposited in the alveolar epithelium illustrated the intensively stained cytoplasm in all infected groups (Figure 25A-G). Moreover, some cells of the pneumocyte type II were prominent with intensively stained cytoplasm in five (Figure 25G) and six (Figure 25H) days groups after infection.

Pulmonary arteriole (Figure 26)

The pulmonary arterioles demonstrated the positive golden brown coloration with various intensities in all of the infected groups from first hour to six days. The mildly stained cytoplasm of the endothelial cells and smooth muscle cells were performed in

the one hour to five days after infection (Figure 26A-G) then became moderate stain in the later groups, six days (Figure 26H) whereas the smooth muscle cells showed mild stain. In addition, the tunica adventitia layer showed intensive golden brown coloration in the one hour to two days after infection (Figure 26A-D) and became moderate stain in the later three to five days group (Figure 26E-G) and mild stain in the six days group (Figure 26H). Moreover, the fine positive intensive line at the apical border of the endothelial cells and in the lumen were demonstrated (Figure 26H).

Tributary of the pulmonary vein (Figure 27)

The tributary of the pulmonary vein, including small vein and venule of all infected groups from first hour to six days demonstrated various intensities of positive golden brown stain. The intensive golden brown coloration was performed in the cytoplasm of the endothelial cells and smooth muscle cells of small veins in first to six hours groups (Figure 27A-B). Later, the mild stain was performed in the cytoplasm of the endothelial cells and smooth muscle cells in the groups of one to two days and moderate stain in three days after infection. Then they became intensive in the group of four days (Figure 27F); moderate in five days (Figure 27G) and mild in six days. Concerning the tunica adventitia, it appeared mild positive stain in all infected group (Figure 27A-B and F-G).

The venule wall showed intensive coloration in all infected groups (Figure 27C-E) except those in the six days group that appeared mild stain (Figure 27H). The lumen of the small vein and the venule were congested with red blood cells and inflammatory cells which demonstrated various intensities of positive golden brown coloration (Figure 27A-H).

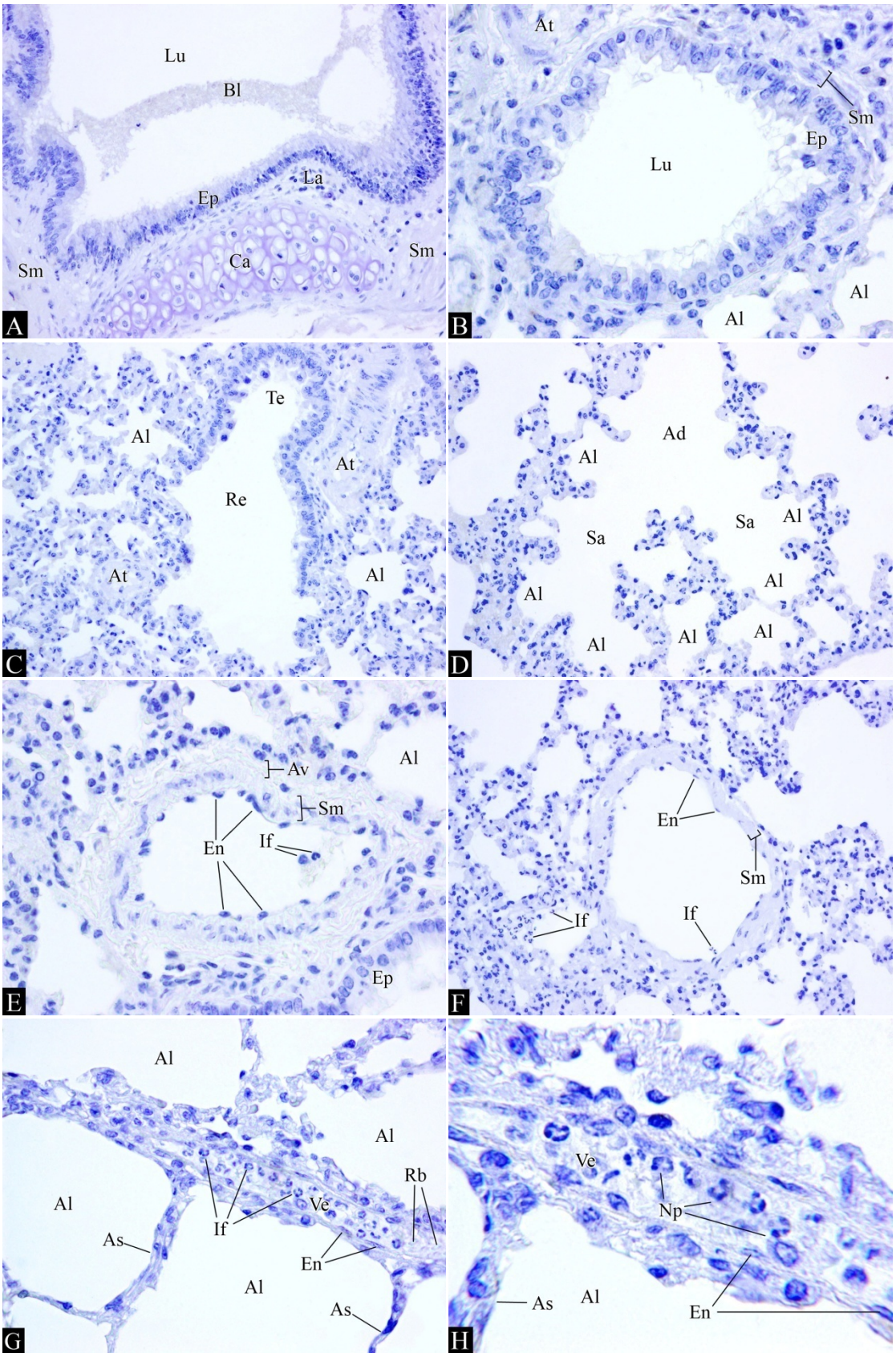
Table 5 The distribution of leptospiral antigen in the lung tissue.

Type of cell	Infection time periods							
	1 hr.	6 hrs.	1 day	2 days	3 days	4 days	5 days	6 days
Bronchiole								
● Epithelium	+++	+++	+	+	++	++	+++	++
● Smooth muscle	+++	+++	+	+	+	+	+	+
● Connective tissue	+++	+++	+++	+++	+	+	++	+
Alveolar epithelium	++	++	+	+	++	++	+	+
Arteriole								
● Endothelium	+	+	+	+	+	+	+	++
● Smooth muscle	+	+	+	+	+	+	+	+
● Adventitia	+++	+++	+++	+++	++	++	++	+
Small vein								
● Endothelium	+++	+++	+	+	++	+++	++	+
● Smooth muscle	+++	+++	+	+	++	+++	++	+
● Adventitia	+	+	+	+	+	+	+	+
Venule wall	+++	+++	+++	+++	+++	+++	+++	+

(-) negative stain, (+) mild positive stain, (++) moderate positive stain and (+++) intensive positive stain

Figure 22 Photomicrographs illustrating the experimental negative control of the lung tissue in the groups of six hours (A and B), one day (C), two days (D and E) and four days (F-H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes. The pictures display various structures of the lung tissue, including the bronchus (A) (Obj 20x); the bronchiole (B) (Obj 40x); the terminal bronchiole (Te) and respiratory bronchiole (Re) (C) (Obj 20x); the alveolar duct (Ad), alveolar sac (Sa) and alveolus (Al) (D) (Obj 20x); the arteriole (E) (Obj 40x); the small vein (F) (Obj 40x); the venule (G and H) (Obj 40x and 100x respectively).

Abbreviation: As, Alveolar septum; Av, Tunica adventitia; Bl, Blood elements; Ca, Hyaline cartilage; En, Nucleus of endothelial cell; Ep, Epithelium; If, Inflammatory cell; La, Lamina propria; Lu, Lumen; Np, Neutrophil; Rb, Red blood cell; Sm, Smooth muscle layer.


A
Lu
Bl
La
Ep
Ca
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Lu
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Al
D
Ad
Al
Sa
Sa
Al
Al
Al
Al
Al
E
Av
Al
Sm
En
If
Ep
F
En
Sm
If
If
G
Al
Al
Rb
Al
Ve
If
As
En
Al
As
H
Ve
Np
As
Al
En

Figure 22

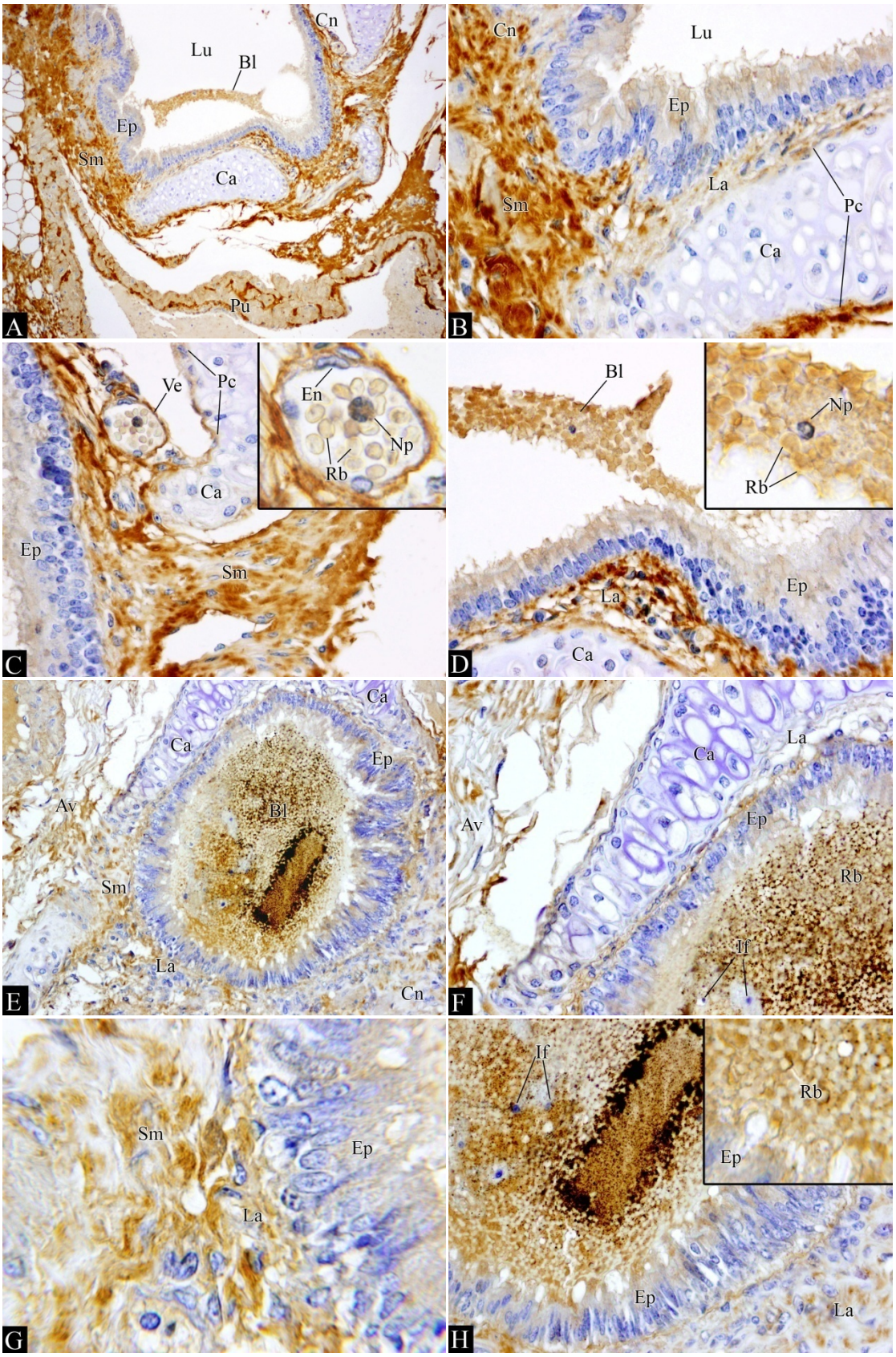
Figure 23 Photomicrographs of the infected lung illustrating the bronchus in the groups of six hours (A-D) and four days (E-H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes.

A-B) The low (A) and high (B) magnification of the bronchus appears mild positive stain in the cytoplasm of the epithelial cell (Ep), moderate in the lamina propria (La) and intensive in the smooth muscle (Sm) and connective tissue (Cn) and blood elements occupying the lumen. Besides, the perichondrium (Pc) demonstrated the moderate to intensive stain. (A, Obj 10x; B, Obj 40x)

C-D) The epithelium of the bronchus displays the mild stain. The lamina propria layer of the bronchus, the wall of venule (Ve) and smooth muscle cells show intensive golden brown coloration. The perichondrium displays moderate (D) and intensive (C) stain. The lumen is congested with red blood cells (Rb) and neutrophils (Np) which appears as mild and intensive positive stain respectively. The lumen of the bronchus reveals blood elements consisting of red blood cells and neutrophils which display intensive and mild positive stain respectively. (Obj 40x, inset 100x)

E-H) The cytoplasm of the epithelium appears mild positive stain while the smooth muscle cells display intensive golden brown coloration. The lamina propria reveals the mild stain in F and intensive in H. The lumen of the bronchus is occupied by the blood elements consisting of red blood cells and inflammatory cells (If). They appear positive stain in various intensity from mild to intensive. (E, Obj 20x; F-H, Obj 40x and inset 100x)

Abbreviation: Av, Tunica adventitia; Ca, Hyaline cartilage; Pu, Pulmonary vein.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Sm
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Sm
La
Cn
F
La
Ca
Av
Ep
Rb
If
G
Sm
Ep
La
H
If
Rb
Ep
Ep
La

Figure 23

Figure 24 Photomicrographs of the infected lung illustrating the bronchioles in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes. (Obj 40x)

A-B) The cytoplasm of the epithelial cell (Ep) and smooth muscle cell (Sm) of the bronchioles appears intensive golden brown stain. Moreover, the alveolar epithelia also perform the intensive stain.

C-D) The mildly positive stained cytoplasm of the epithelia and smooth muscle of the respiratory bronchiole (Re) are observed. The connective tissue stroma (Cn) shows intensive stain.

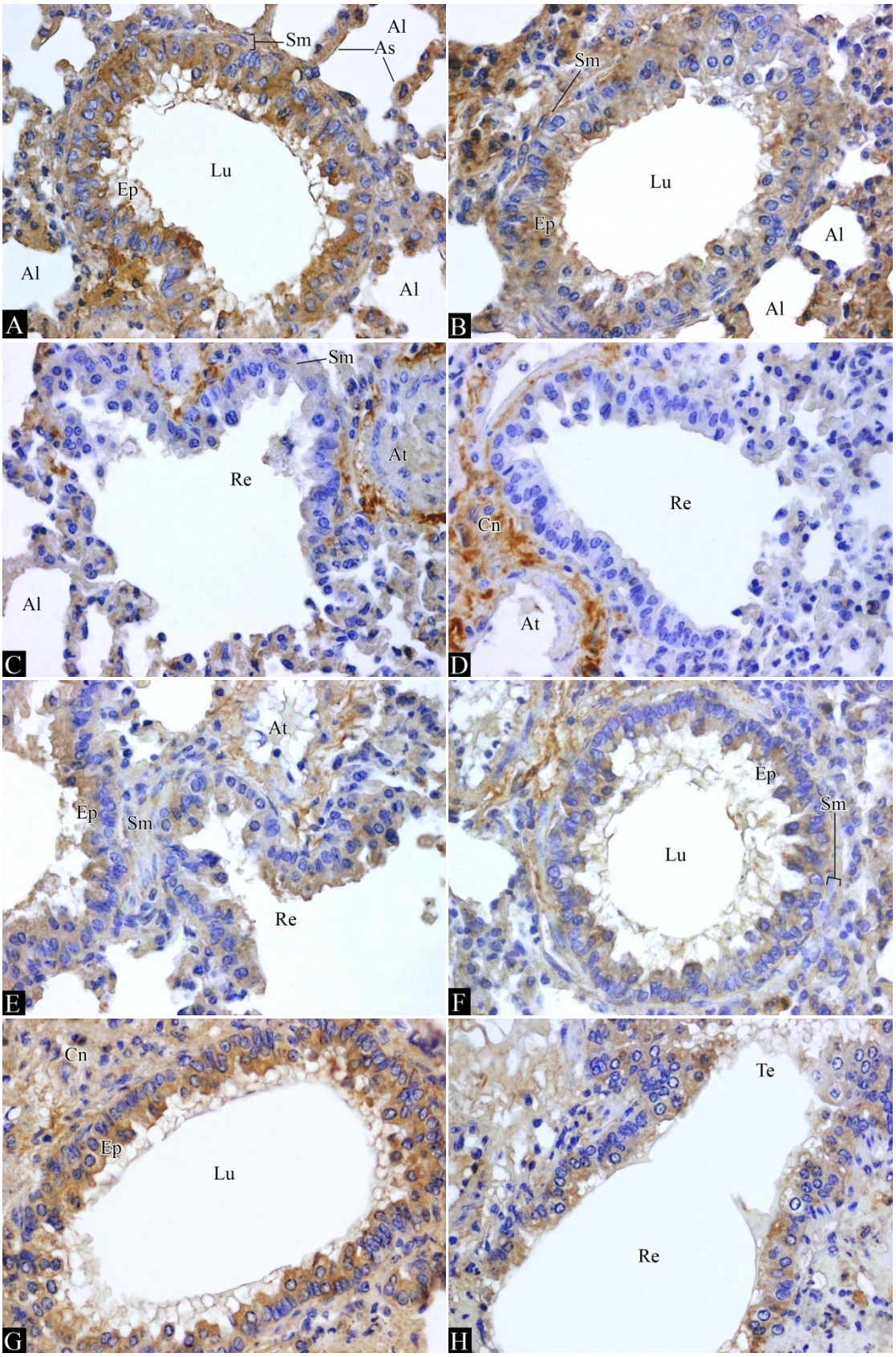
E) The respiratory bronchiole appears moderate golden brown coloration in the cytoplasm of the epithelial cells while mildly positive stain is observed in smooth muscle cell.

F) The cytoplasm of the epithelial cells of the bronchiole appears moderate positive stain while the smooth muscle cells show mild golden brown stain.

G) The cytoplasm of the epithelia of the bronchiole performs the intensive golden brown coloration. The connective tissue stroma showed the moderate stain.

H) The terminal bronchiole (Te) and respiratory bronchiole illustrate moderately positive stained cytoplasm of the epithelial cells.

Abbreviation: As, Alveolar septum, At, Arteriole; Lu, Lumen.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At
D
At
Ep
Sm
Re
E
Ep
Sm
Lu
F
Cn
Ep
Lu
G
Te
Re
H

Figure 24

Figure 25 Photomicrographs of the infected lung illustrating the alveolar epithelium in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes. (Obj 40x, inset100x)

A) The infected lung tissue demonstrates the alveolar epithelium and neutrophils (Np) which appear moderate and intensive golden brown coloration respectively. Some alveolar epithelia show the intensively stained fine filamentous structures attaching at the apical border of the cell (arrow head).

B) The alveolar epithelia reveal the moderate stain while the neutrophils are intensive stain.

C-D) The alveolar epithelia demonstrate mildly positive stained cytoplasm. The neutrophils depositing on the alveolar epithelia display moderate (C) to intensive stain (D).

E-F) The cytoplasm of the alveolar epithelium appears moderate golden brown stain. The cytoplasm of neutrophils show intensive positive stain.

G-H) The alveolar epithelia reveal the mildly positive stained cytoplasm. The cytoplasm of the type II pneumocytes appear intensive golden brown coloration (H) while some cytoplasm of neutrophils show mild to intensive golden brown positive stain (G).

Abbreviation: Al, Alveolus; As, Alveolar septum; Ve, Venule.

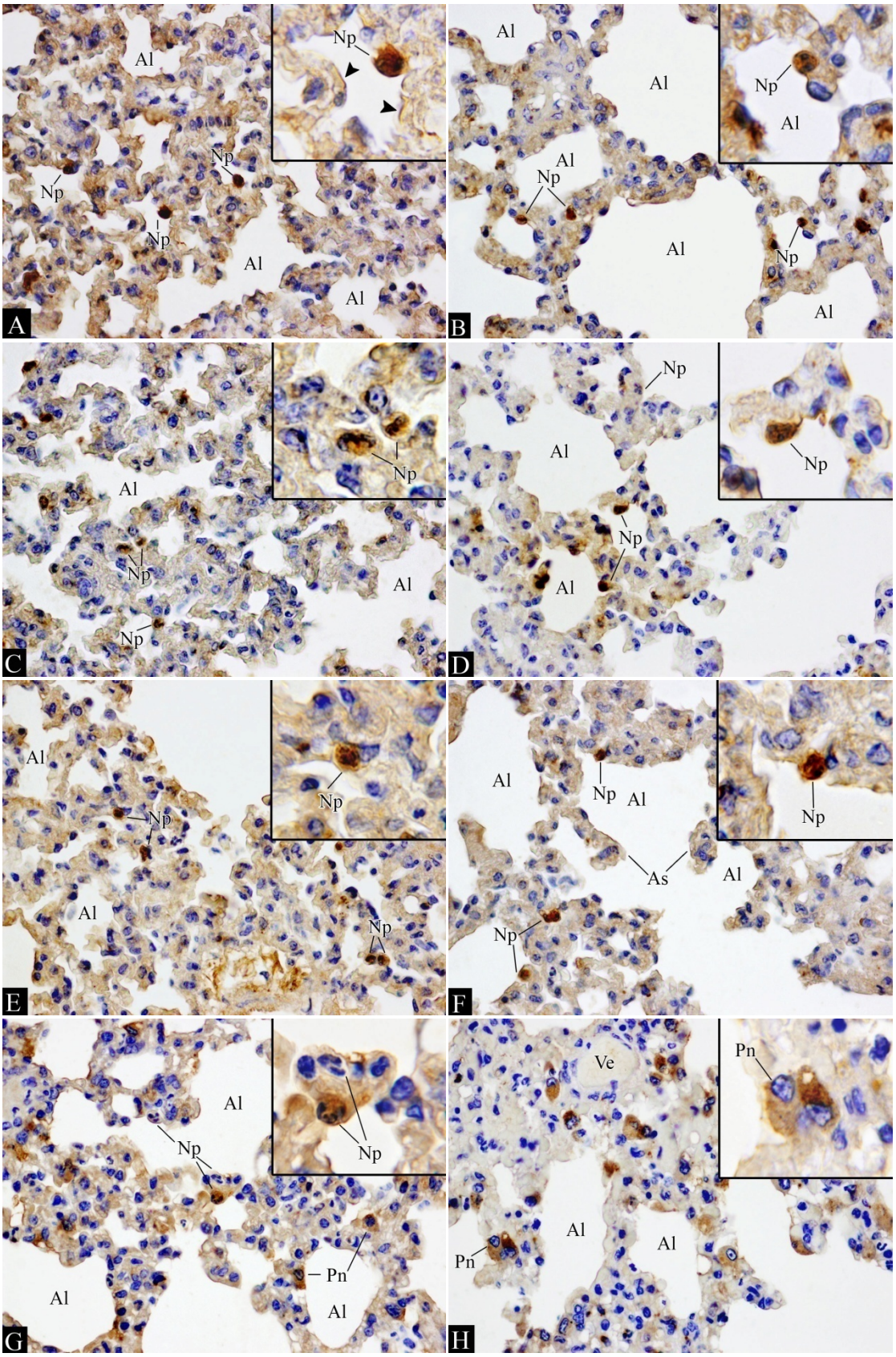


Figure 25

Figure 26 Photomicrographs of the infected lung tissue illustrating the pulmonary arterioles in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes.

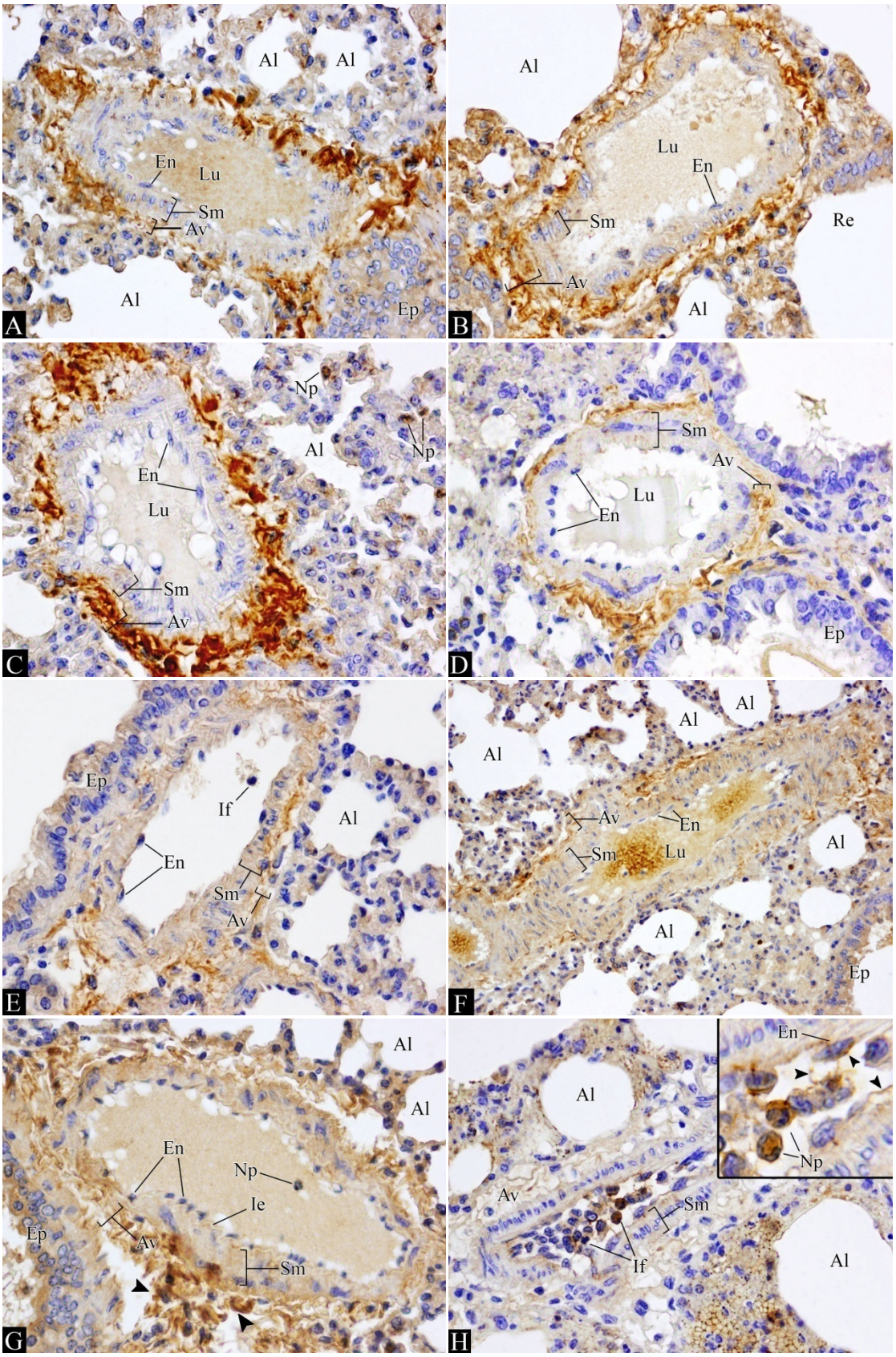
A-D) The cytoplasm of the endothelial cells (En) and smooth muscle cells (Sm) of the arteriole show mild positive stain while the tunica adventitia (Av) demonstrates intensive coloration. (Obj 40x)

E-F) The mildly positive stained cytoplasm of the endothelial cells and the smooth muscle cells are demonstrated. The tunica adventitia of the arteriole shows moderate stain (E, Obj 40x and F, Obj 20x).

G) The cytoplasm of the endothelial cells and the smooth muscle cells demonstrates mild positive stain. The tunica adventitia appears moderate to intensive (arrow head) golden brown stain. (Obj 40x)

H) The moderately positive stained cytoplasm of the endothelial cells are observed while the smooth muscle cells and the tunica adventitia are mild stain. The lumen of the arteriole is filled with inflammatory cells (If) presumed the neutrophils reveal intensive stain. The golden brown fine line attaching at the border of the epithelial cells and locating in the lumen (arrow head) are also demonstrated. (Obj 40x, inset 100x)

Abbreviation: Al, Alveolus; Ep, Epithelium of bronchus; Ie, Internal elastic lamina; Lu, Lumen; Re, Respiratory bronchus.


A
Al
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En
Lu
Sm
Av
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Ep
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En
Re
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F
Al
Al
Al
Av
En
Sm
Lu
Al
Al
Ep
G
Al
Al
En
Np
Ie
Ep
Av
Sm
H
En
Al
Np
Av
Sm
If
Al

Figure 26

Figure 27 Photomicrographs of the infected lung illustrating the tributary of the pulmonary vein in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans* serovar pyrogenes.

A-B) The small vein shows intensive golden brown stain in the endothelial cytoplasm (En) and the smooth muscle cells (Sm). The red blood cells (Rb) occupying in the lumen and tunica adventitia (Av) showed the mild stain. (Obj 20x)

C-E) The venule wall demonstrates intensive positive stain area. The mildly positive stained neutrophils (Np), monocyte (Mn) and red blood cells (Rb) are demonstrated in the lumen. (Obj 40x)

F) The intensively stained cytoplasm of the endothelial cells and the smooth muscle cells are observed in the small vein while the tunica adventitia shows mild stain. The inflammatory cells (If) and red blood cells occupying in the lumen appear mild stain. (Obj 20x)

G) The endothelial cytoplasm and the smooth muscle cells demonstrate moderate stain and the tunica adventitia appear mild stain. The various intensities of positive stained red blood cells and inflammatory cells are expressed in the lumen. (Obj 20x)

H) Wall of the venule shows mild golden brown stain. The mildly stained red blood cells and inflammatory cells congest in the lumen. Some inflammatory cells show moderate golden brown stain (arrow head). (Obj 40x)

Abbreviation: Al, Alveolus.

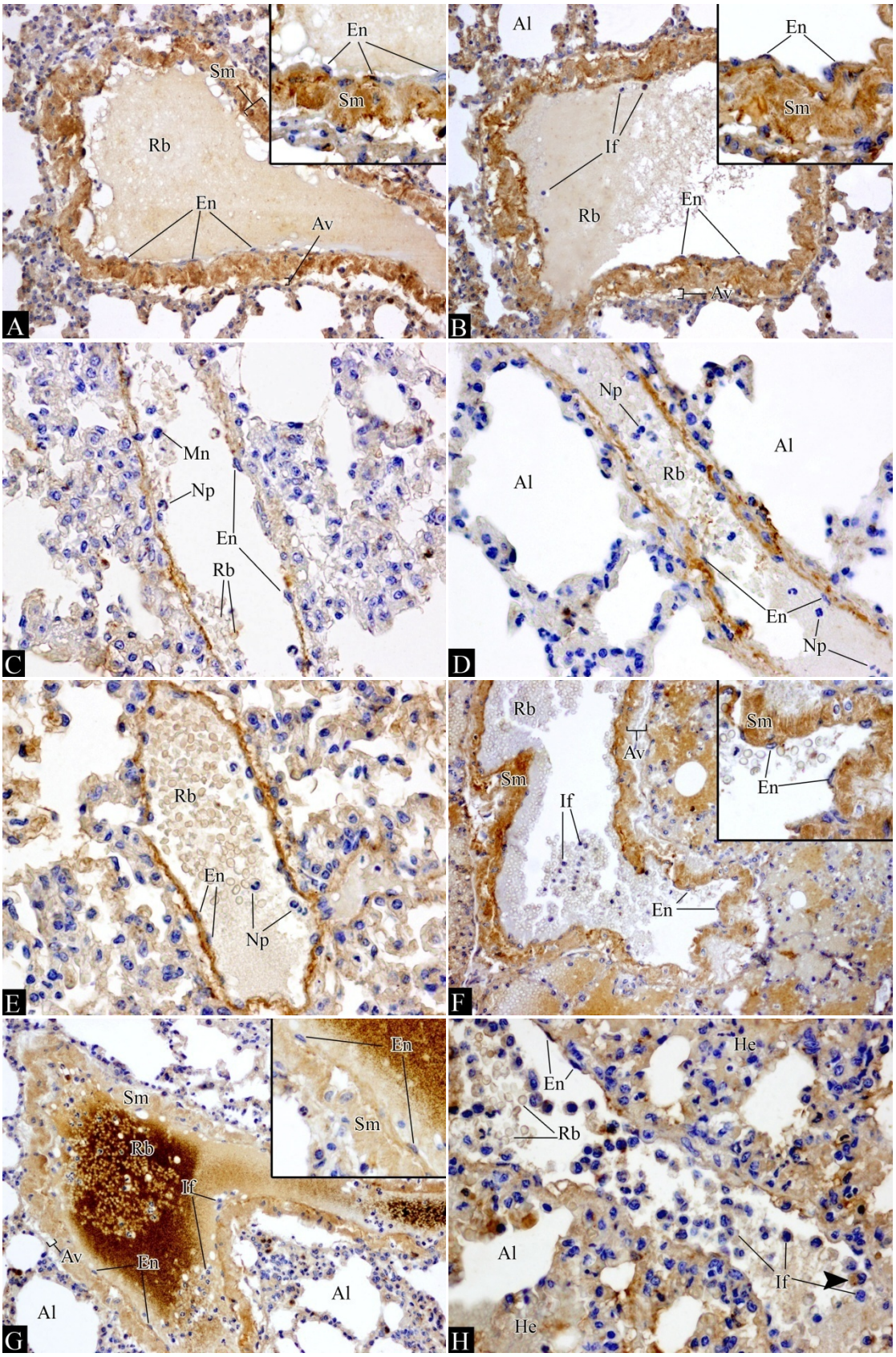
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Figure 27

CHAPTER VI
DISCUSSIONS

The study of organs of the infected hamsters, including the liver, lung and spleen demonstrated the positive golden brown stain of various intensities in the sections. The golden brown coloration localized in these tissues was presumed to be the leptospiral antigen or even the leptospires themselves.

Liver

The liver was one of the major infected organs for leptospirosis (Zhang *et al*., 2007). During the septicemic phase of the disease, the leptospires and/or their antigen product spreaded throughout the host body by the blood stream (Alves *et al*., 1987). The portal area showed a number of inflammatory cell infiltration which indicated the infection (Pilakasiri *et al*., 2001) and corresponded to this study that displayed the leptospiral antigens detected at luminal surface of the portal triad, including the bile duct, hepatic artery and portal vein. The mildly stained cytoplasm of the epithelial cells of the bile duct, smooth muscle cells of the hepatic artery as well as endothelial cells of the hepatic artery and portal vein were illustrated in all experimental groups. Corresponding to the study of Alves *et al*. (1987) reported that the leptospiral antigens depositing on the endothelial lining of the dilated portal vein as well as the thin thread like filaments were observed in the portal triad, closed to a bile duct of human liver leptospirosis

In addition, this study presented the leptospiral antigen in the cytoplasm of the endothelial cells of the central vein with different intensities of golden brown coloration. The luminal contents also exhibited intensive and moderate stain in different groups after infection. This appearance might give the reason why vascular damage occurred in leptospirosis. De Brito *et al*. (1979) reported the vascular lesions were induced by the toxic of pathogenic leptospires.

The cytoplasm and boundary of the hepatocytes appeared the leptospiral antigen depositing nearby the portal area in the early groups of infection, and in the late groups they were found in the distance between the portal area and central vein. This characteristic was observed with various staining intensities from mild to intensive golden brown coloration and might due to the direction of the blood circulation from the portal area to the central vein. These antigens found in the cytoplasm of hepatocytes might give the reason why the degenerative changes were observed in the study of Pilakasiri *et al*. (2001). The study of Pilakasiri *et al*. (2001) studied the hamsters infected with *L. interrogans* serovars pyrogenes and reported that the enlarged and vacuolated hepatocytes were related to the cloudy swelling. Additionally, hepatocellular necrosis was found scattering throughout the hepatic lobules which were a sign of hepatocellular damaged and disorganization of the liver structure and function. The ultrastructure of infected liver demonstrated edematous microvillus, irregular, less electron dense free surface of the hepatic cells and total or partial disappearance of the microvilli of bile ductules (De Brito *et al*., 1966 and 1967). The human liver cells were damaged after 24 hours infection with *L. interrogans* serovar Lai (Zhang *et al*., 2008). Saglam *et al*. (2008) reported that leptospiral antigens were located in the cytoplasm of the hepatocytes in the portal regions of ovine abort fetuses. The equine abort fetus livers displayed the abundant leptospiral antigens (Szeredi and Haake, 2006).

The previous study reported that the intact and well-shaped leptospires were found in the liver of infected marmoset monkeys by immunofluorescence (Pereira *et al*., 2005). In addition, many leptospires were found, especially in the space of Disse and the dilated intercellular spaces by the electron microscopic study (van de Ing and Hartman, 1986 and De Brito *et al*., 1966). The indirect immunoflorescent staining presented leptospires between hepatocytes (Merien *et al*., 1998). The silver and immunohistochemical staining showed the large number of leptospires in infected livers of guinea pig with *L. interrogans* serovar copenhageni (Nally *et al*., 2004). These appearances confirmed that the leptospires invaded to the hepatic parenchyma which corresponded to the location of leptospiral antigens found in this study.

In the present study revealed the intensive golden brown coloration along the boundary of the hepatocytes and mild stain in the cytoplasm. This phenomenon was

found in the group of five and six days post infection and might be the cause of hepatocytes losing contact with each other making discohesion of the hepatic cord. As a result, the hepatocytes lose of E-cadherin according to the study of De Brito *et al*., (2006). De Brito *et al*., (2006) study in the leptospirosis patient, expressions of E-cadherin in liver cells were irregular. In the area of liver plate disarray, no any expressions of E-cadherin were displayed in adjacent to hepatocytes. The dissemination of leptospires was observed in acutely infected guinea pigs in which large numbers of leptospires were seen in the liver and kidney (Nally *et al*., 2005).

Hepatic sinusoid demonstrated depositing leptospiral antigens in all groups of infection studied. It corresponded with the typical leptospiral wavy forms that were observed in the sinusoid of the liver of equine abort fetuses by immunohistochemistry (Szeredi and Haake, 2006). The histopathology showed congested sinusoids and there were diffused focal haemorrhages in autopsied human leptospirosis (Arean, 1962) and in infected hamsters (Pilakasiri *et al*., 2001). Disruptions of the sinusoidal endothelial cells were marked (van de Ing and Hartman 1986). A massive destruction of extravascular red blood cells liberated by haemorrhage diathesis, appeared to be the main cause of jaundice (Higgins and Cousineau, 1976).

The red blood cells in hepatic sinusoid and haemorrhagic area displayed the positive stain in this study. These observations might due to red blood cells were covered with the leptospiral antigens. Yuri *et al*. (1993) demonstrated the number of leptospires that moved toward hemoglobin than the distilled water in their experiment. The toxin of leptospires induced hemolysis on sheep erythrocytes and exhibited discrete membrane disruptions and cellular swelling (Lee *et al*., 2000). The electron microscopic study demonstrated deformed erythrocytes and the breakage of their plasma membrane in the hepatic sinusoid (Yang *et al*., 2006). The leptospiral infection had been occurring for cytotoxic factor to appear in the blood. The production of cytotoxic factor in the blood and plasma might be a form of leptospiral infections (Knight *et al*., 1973). The previous study that described above might confirm that the leptospire themselves and/or their antigens contacted or attached to red blood cells.

The inflammatory cell mainly neutrophils in the portal area, hepatic sinusoid and central vein displayed leptospiral antigens in the cytoplasm at one to six days post infection. This finding corresponded with the report of Pilakasiri *et al*. (2001) which

observed the inflammatory cell infiltration in the liver parenchyma which indicated the infection. The prime function of the neutrophil was phagocytosis and digestion of phagocytosed material such as bacteria (Rhodin, 1974). That was why the leptospiral antigens were founded in the cytoplasm of neutrophils.

The neutrophil were not the only inflammatory cell that was found in the hepatic parenchyma but also the Kupffer cell. Therefore, the Kupffer cell might engulf the leptospires and/or their antigens that made their cytoplasm appeared positive stain. Because of their highly phagocytic activity, their cytoplasm contained a vary number of primary and secondary lysosome (Rhodin, 1974). The liver of the human leptospirosis exhibited cytoplasmic antigen depositing in the Kupffer cells (Alves *et al*., 1987) and the macrophage in the portal region of ovine abort fetuses (Saglam *et al*., 2008). The ultrastructural study infected liver illustrated enlargement of Kupffer cells with irregular shape and with irregular dense bodies in the cytoplasm (De Brito *et al*., 1966 and 1967).

Spleen

The capsule and the trabeculae of the spleen in all experimental groups displayed the various intensities of positive stain. This staining pattern reflected the leptospiral antigen or even their leptospires themselves from the blood supply could invade and deposit in the capsule and trabeculae. This should be agreed with the work of Arean (1962) which reported that the large spleens of the seven leptospirosis patients showed the capsule that was tense reddish purple and shiny on gross observation.

The white pulp receives blood supply from the central artery. Therefore, in this study, the endothelial cells and the smooth muscle cells of the central artery appeared various intensities of positively stained cytoplasm in all experimental groups due to the circulating leptopires or their antigens in the blood supply. Szeredi and Haake (2006) reported that the typical leptospiral wavy forms were found in the blood vessels of various equine abort fetal organs. The histological observations of infected guinea pigs with *L. interrogans* serovar icterrohaemorrhagiae revealed that the main lesion was a severe injury of vasculature of mainly arteries, arterioles and capillary. Most endothelial cells were affected or destroyed and the muscular fibers of the arteries and

arterioles were injured. The arterial musculatures were more vacuolized (Higgins and Cousineau, 1977).

Pathogenic leptospires possessed a number of the protein antigens that were expressed during infection of mammalian hosts and become targets for the host immune response (Guerreiro *et al*., 2001). In this study, the plasma cells in the white pulp were positive mild stain in all groups of infection and the moderately and intensively stained cytoplasm were found in the late groups. This characteristic might indicate that leptospires or their antigens became increasing as the time of infection continued which corresponded with the increasing severity of the pathology of the hamsters spleen infected with *Leptospira interrogans* serovar pyrogenes in the later groups (Muensoongnoen *et al*., 2006). The plasma cells were the main source of the antibody synthesis. Therefore, these cells represented an important link of the immunological defense system and increased greatly in number under conditions of chronic inflammation (Rhodin, 1974). Several proteins of leptospires were identified as targets of the humoral response during natural infection. In both acute and convalescent phases of illness, antibodies to proteins were exclusively immunoglobulin G (IgG), whereas antibodies to lipopolysacharide were predominantly IgM (Guerreiro *et al*., 2001). The autopsied leptospirosis patients revealed abundant plasma cells (Arean, 1962).

The leptospiral antigens observed in the venous sinus in the red pulp expressed the mildly stained cytoplasm of the endothelial cells in all experimental groups. During the septicemic phase of the disease, the leptospires and/or their antigens spread throughout the host body by the blood stream (Alves *et al*., 1987). The pathogenesis of the haemorrhagic diathesis in experimental leptospirosis of the guinea pig was described by the paucity of the microorganism in the vicinity of the lesions were in accordance with the toxic genesis postulated for the vascular damage in the leptospirosis. It was suggested that the vascular lesions induced by leptospirosis began with the increased permeability prior to endothelial cell necrosis (De Brito *et al*., 1979). Corresponding to Muensoongnoen *et al*. (2006) reported that the multiple haemorrhagic areas scattered throughout the red pulp of the infected spleen. Moreover, in this study demonstrated the aggregation of the intensively stained fine filamentous form which should be assumed to be the leptospires themselves. Anyhow, the indirect

immunofluorescence for leptospires on paraffin section showed mainly extensive coarse granules in the red pulp (van der Ingh and Hartman, 1986).

The leptospiral antigen was also found in the cytoplasm of the macrophage with mild stain in all groups post infection except the moderate stain in six day group. This cell was prominent in the late group after infection. The macrophages represent an essential part of the defending system of the mammal against the invading of the microorganism, antigen (foreign protein) and inert foreign matter (Rhodin, 1974). Muensoongnoen *et al*., (2006) founded the brown hemosiderin granules in the cytoplasm of the macrophage in infected hamsters with *L. interrogans* serovar pyrogenes. The TEM analysis confirmed the formation of the leptospire containing phagosomes in murine monocyte derived macrophage (Liu *et al*., 2007). The immunoperoxidase staining technique detected leptospiral antigen in the splenic tissue of the ovine abort fetuses and demonstrated the leptospiral antigen in the cytoplasm of the macrophages throughout the parenchymal tissue and were found in the other tissue, such as lung, liver and kidney (Saglam *et al*., 2008).

The inflammatory cells mainly neutrophils and lymphocytes were found in the infected splenic parenchyma and sinusoid (Muensoongnoen *et al*., 2006). The neutrophils increased and formed discrete foci scattering throughout the parenchyma (Arean, 1962). The infected guinea pig with *L. interrogans* serovar copenhageni presented the reticuloendithelial system activation with increased hemophagocytosis (Nally *et al*., 2004). These pathological finding supported the various intensities of stained cytoplasm of the neutrophil in the present study. The mildly stained cytoplasm of the neutrophil was observed in all groups but the prominent intensive stain was found in the last group of infection. The prime function of the neutrophil was phagocytosis and digestion of the phagocytosed material, such as bacteria (Rhodin, 1974). In this study, the neutrophil were found prominent in the red pulp nearby the marginal zone and in the marginal zone in one day group and the number was increasing as the time infection went on. These phenomenon might be described by the marginal zone was designed to screen the systemic circulation for antigens and pathogens and played an important role in antigen processing (Cesta, 2006).

The red blood cells in both haemorrhagic area and lumen of blood vessels in the red pulp revealed the different staining intensities. These cells might be covered with

leptospiral antigen circulating in the blood stream. The experiment of Yuri *et al*. (1993) demonstrated the more number of leptospires that moved toward the uninoculated tops containing hemoglobin than the uninoculated tops containing distilled water. Knight *et al*., 1973 suggested leptospirosis had been occurring from the cytotoxic of leptospires that appeared in the blood and plasma. Therefore, it should be explained why the leptospiral antigens preferred to attach the red blood cells in order to circulate in the blood stream.

Lung

The cytoplasm of the epithelia of the bronchus, bronchiole, terminal bronchiole and respiratory bronchioles displayed the positive stain in all infected groups. This phenomenon indicated they should be invaded by leptospires or their antigens. Since, after leptospires had penetrated in to the body of the host, they lived in the blood stream and they had ability to adhere themselves to endothelial cells and then penetrate into the tissue (Barbosa *et al*., 2006). Correspoding to the study of Thomas and Higbie (1990) reported that the pathogenic leptospires were found both attaching to and within the epithelial and endothelial cells tested in the tissue culture. These appearances were confirmed by SEM and TEM. Arean (1961) observed the fatal human leptospirosis and reported the pathology of the respiratory system, including hyaline fibrin membrane, incipient bronchitis and bronchopneumonia. In addition, intrabronchial heamorrhages dispersed throughout the pulmonary parenchyma were observed by Higgins and Cousineau (1977). These leptospiral antigens existing in the tissue might be the etiology of the pathology of the organs.

In addition, the alveolar epithelium presented the positive golden brown stain in all infected groups. This finding indicated that the leptospiral antigen or even the leptospires themselves could contact and come inside the epithelial cells. The leptospiral antigen that presented in those alveolar epithelial cells might give the reason of the histopathological changes observed in the lung tissue in the study of Pilakasiri *et al*. (2001). The study of Pilakasiri *et al*., (2001) which studied in the hamsters inoculated with *Leptospira interrogans* serovar pyrogenes reported that the intraalveolar capillaries were distended with red blood cells. Most area of the lung demonstrated the interstitial and intra-alveolar haemorrhage as well as the alveolar

septum thickening by accumulation of the inflammatory cells consisting of an increase number of neutrophils, lymphocytes, plasma cells and macrophages. In all infected guinea pigs with *L. interrogans* serovar copenhageni in the experiment of Nally *et al*., (2004) showed significant microscopic haemorrhage in the lung. The alveolar septum increased the number of mononuclear cells and occasionally neutrophils as commonly founded in the Marmoset monkeys infected with the same serovar (Pereira *et al*., 2005). The ultrastructure of the alveolar epithelium illustrated bleb formation and the cytoplasm appeared swollen and protruded into the alveoli. (Miller *et al.,* 1974). Moreover, the lung of human leptospirosis collected after death showed prominent macrophages, lymphocytes, plasmocytes and tumefaction of the endothelial cells. The TEM demonstrated tumefaction, increased numbers of pinocytic vesicles and presence of giant dense bodies in the cytoplasm of the endothelial cells (Nicodemo *et al*., 1997).

In this study, the fine filamentous forms were observed attaching at the apical border of the alveolar epithelial cells. These structures might be leptospires or their antigens. The previous study, Nicodemo *et al*., (1997) demonstrated filamentous leptospiral antigen attached to the endothelial cells of the septal capillary and granular form in the endothelial cell of the interalveolar septum by immunohistochemical technique. In addition, the immunohistochemical study was performed in aborted equine fetuses by Szeredi and Haake (2006) which demonstrated the typical leptospiral wavy forms aggregated in the alveoli. In the five and six days groups post infection, the pneumocyte type II was prominent with intensively golden brown stained cytoplasm, whereas the another alveolar epithelial cells displayed mild positive stain. These findings might correspond to report of Keig *et al*., (2001) that studied gram negative bacteria, *Burkholderia cepacia* strain C1359 in human. They found that this organism could invade pneumocyte type II and intracellularly multiply.

The previous pathological study of Pilakasiri *et al*., (2001) showed the haemorrhage in lung tissue and thickening of alveolar septum by accumulation of the inflammatory cells consisting of an increase number of neutrophils, lymphocytes, plasma cells and macrophages. In the present study, the positive golden brown stain was performed in the inflammatory cells which were mainly neutrophil in the alveolar septum, in lumen of blood vessels and in the haemorrhagic area. Normally the neutrophils were interacted with chemotactic agents then migrated to the site invaded

by microorganism. They stopped migrating and preparing for their passage through the endothelium of the postcapillary venule to enter the tissue (Gartner and Hiatt, 2001). After that they ingested the particles or the antigens by local invagination or by pseudopods extension (Bloom and Fawcett, 1986). These results might give the reason why the positive stain was observed in the cytoplasm of neutrophils. However, the TEM showed very few pathogenic leptospires were ingested within human neutrophil, but many pathogenic leptospires attached to the surface of the neutrophils in the experimental of Wang *et al*., (1984). Moreover, the immunoperoxidase studies demonstrated leptospiral antigens were located in the cytoplasm of the macrophage in the interalveolar, interlobular septum and alveoli (da Silva *et al*., 2002; Saglam *et al*., 2008)

The red blood cells in haemorrhagic area and in the lumen of blood vessels revealed the positive stain in this study. This appearance might be because the red blood cells were covered with leptospiral antigens. Because of the red blood cells were composed of mainly hemoglobin (Gartner and Hiatt, 2001). Corresponding to the experimental of Yuri *et al*., (1993) illustrated the pathogenic leptospires preferred to move toward the uninoculated top containing the hemoglobin than the uninoculated top containing distill water in the U-shaped polypropylene tube. Knight *et al*., (1973) reported the whole blood and plasma from animals in the acute stage of leptospirosis contained toxic substances in circulating in the blood and *L. interrogans* serogroup icterrohaemorrhagie was identified in the blood of one patient (García *et al*., 2000) and agglutination tests revealed antibodies to *L. icterohaemorrhagiae* and *L. australis* (Turhan *et al*., 2006). In addition, Lee *et al*., (2002) showed sheep erythrocyte membrane formed pore when treated with the *Leptospira* hemolysin SphH by TEM study.

According to the previous studies, the lung tissues were taken the leptospires or their antigen from the blood supply. The component of the wall of pulmonary arterioles and the tributary of pulmonary vein, including the endothelial cells, smooth muscle cells and tunica adventitia appeared positive stain in this study. These appearances were clearly known that the leptospiral antigens were located in the blood vessels. Pulmonary haemorrhage might occur because the endothelial cells showed swollen and desquamation with capillary rupture (De Brito *et al*., 1979). In addition,

the ultrastructure of the lung showed varying degree of endothelial and epithelial cells injury, characterized by swollen and vacuolated cytoplasm (Nally *et al*., 2004). The bleb formation was seen in the capillary endothelium (Miller *et al*., 1974) and arterial musculature was more vacuolized (Higgins and Cousineau, 1977). Miller *et al*., (1974) suggested these appearances should respond to toxic substance of leptospires.

In addition, the immunohistochemistry and PAS/Steiner silver stain of the infected hamster lung tissue revealed a highly density of *L. interrogans* serovar Pomona strain 11000-74A within the lumen of a blood vessel by Matsunaga *et al*., (2006). Szeredi and Haake (2006) reported the typical wavy forms aggregated in the lumen of blood vessel in various organs of aborted equine fetuses. These findings confirmed that the leptospires and/or their antigens were circulating in the blood.

The tunica adventitia in all infected groups showed the prominent positive stain more than the endothelial cells and the smooth muscle cells. These appearances might be described in the way that leptospires penetrated into the tissue and contact with extracellular matrix involved in the initial steps leading to infection (Babosa *et al*., 2006). The tunica adventitia was composed mostly fibroblasts, collagen type I and elastic fibers (Gartner and Hiatt, 2001). In the previous studies, Chirathaworn *et al*., (2007) reported the pathogenic leptospires could bind to various type of extracellular matrix and preferred fibronectin than collagen and laminin. Barbosa *et al*., (2006) demonstrated the leptospiral protein of *L. interrogans* serovar copenhageni exhibited attachment to laminin. Lin and Chang (2007) suggested the leptospiral immunoglobulin-like protein (LigB) contributed to high affinity binding to N-terminal domain and gelatin domain of fibronectin, laminin and fibrinogen and mediated leptospires binding to host cells. A new leptospiral protein that called Lsa21 (Leptospiral surface adhesion, 21 kDa) exhibited binding to extracellular matrix. The stronger interaction in laminin, collagen type I and plasmafibronectin were observed by Atzingen *et al*., (2008).

Brown *et al*. (2003) reported that the leptospiral materials in postmortem specimens were collected from eight patients who died of leptospirosis by leptospiral culture, direct immunofluorescence and the PCR. These results suggested that in the acute and/or fatal leptospirosis, the pathogenesis of the pathologic features were

related to the presence of the leptospires in the tissues. These informations correspond to the present study that found leptospiral antigens in the pathologic areas.

CHAPTER VII
CONCLUSION

Leptospirosis is a bacterial zoonosis disease caused by spirochetes in the *Leptospira* genus that can affect humans and a wide range of animals. This disease is characterized by the development of vasculitis and endothelial cell damage. The histopathology is most marked in the liver, kidneys, heart and lung. On the other hand, other organs may also be affected according to the severity of the individual infection. The immunoperoxidase stain in the previous study showed deposit of leptospiral antigen serovar icterrohaemorrhagiae in the cytoplasm of endothelial cells of blood vessels in the infected human liver and kidney. However, leptospiral antigens serovar pyrogenes causing outbreak in Northeast of Thailand have not been localized in the infected organs. In the present study attempt to reveal these antigens in the livers, spleens and lung.

Leptospira interrogans serovar pyrogenes was isolated from a febrile patient who has clinical leptospirosis in Burirum Province. These microorganisms were cultured and used in this study. Six control hamsters injected intraperitoneally with 0.5 ml of PBS were served as the control group. The other 24 hamsters were the experimental groups. They were injected intraperitoneally with 0.5 ml of PBS that contained 1×10^8 leptospires per milliliter. Three hamsters of the control group were sacrificed on day 2 and the last three on day 5. The 24 infected hamsters were sacrificed of 3 each at 1 hour, 6 hours and on day 1, 2, 3, 4, 5, and 6 after injection respectively. The livers, spleens and lungs of all the sacrificed animals were removed and processed for immunohistochemical study of the tissue sections by the indirect immunoperoxidase staining technique. Primary rabbit antibody specific for *L. interrogans* serovar pyrogenes (batch # 303, Royal Tropical Institute KIT Biomedical research, The Netherlands.) and rabbit ABC staining system kit: sc-2018, Santa Cruz Biotechnology, Inc was applied.

All infected organs demonstrated the positively golden-brown stained areas which were presumed to be the leptospiral antigens in the sections. Regarding to the liver, leptospiral antigens were detected at luminal surfaces of the portal triad, including the bile duct, hepatic artery and portal vein. The mildly stained cytoplasm of the epithelial cells of the bile duct, smooth muscle cells of the hepatic artery as well as endothelial cells of the hepatic artery, portal vein and central vein were illustrated in all experimental groups. This finding might be clarified that the leptospires and/or their antigen product spreaded throughout the host body by mean of the blood stream. Therefore, the damaged hepatocytes might relate to the existing leptospiral antigens. The cytoplasm and boundary of the hepatocytes nearby the portal area appeared the leptospiral antigen depositing in the early groups of infection and in the late groups these antigens were also found in the hepatocytes locating in the distance between the portal area and central vein. This characteristic was observed with various staining intensities from mild to intensive golden brown coloration and might due to the direction of the blood circulation from the portal area to the central vein. In addition, hepatic sinusoid, their contents and Kupffer cell demonstrated depositing leptospiral antigens in all groups of infection studied.

Concerning the spleen, their capsules and the trabeculae in all experimental groups displayed various intensities of positive stain. The staining pattern reflected the leptospiral antigens or even their leptospires themselves from the blood supply could invade and deposit in the capsule and trabeculae. The white pulp receives blood supply from the central artery. Therefore, in this study, the endothelial cells and the smooth muscle cells of the central artery appeared various intensities of positively stained cytoplasm in all experimental groups due to the circulating leptopires or their antigens in the blood supply.

The plasma cells in the white pulp were mild positive stain in all groups of infection except one hour group and the moderately and intensively stained cytoplasm were found in the late groups. This characteristic might indicate that leptospires or their antigens became increasing as the time of infection continued which corresponded with the increasing severity of the pathology of the hamsters spleen infected with *L. interrogans* serovar pyrogenes. The plasma cells were the source of the antibody synthesis, therefore, these cells represented an important link of the

immunological defensive system and increased greatly in number under conditions of chronic inflammation.

Regarding to the red pulp, the endothelial cytoplasm of the venous sinus expressed mild stain. This appearance might be confirmed that the damaged endothelial cell could be the cause of haemorrhagic diathesis found in the infected hamsters. Additionally, the leptospiral antigens were also found in the cytoplasm of the macrophages with mild stain in all post infection groups except the moderate stain in the six day group. This cell was prominent in the late groups after infection. The macrophages might engulf leptospires and/or their antigens because they represented an essential part of the defending system of the mammal against the invading of the microorganism, antigen (foreign protein) and inert foreign matter.

In the lung, the cytoplasm of the epithelia of the bronchus, bronchiole, terminal bronchiole and respiratory bronchiole displayed the positive stain in all infected groups. This appearance indicated that they should be invaded by leptospires or their antigens. Since, after leptospires had penetrated in to the body of the host, they lived in the blood stream and had ability to adhere themselves to endothelial cells and then penetrated into the tissue.

Concerning to the alveolus, the alveolar epithelium presented the positive golden brown stain in all infected groups. This finding indicated that the leptospiral antigens or even the leptospires themselves could contact and then come into the epithelial cells. The leptospiral antigens that were presented in those alveolar epithelial cells might give the reason why the histopathological changes were observed in the lung tissue.

The lung tissue suffered from leptospires and/or their antigens in the blood circulation because the component of the wall of pulmonary arterioles and the tributary of pulmonary vein, including the endothelial cells, smooth muscle cells and tunica adventitia appeared positive stain in this study. These appearances were clearly known that the leptospiral antigens were located in the blood vessels. Pulmonary haemorrhage might occur because the endothelial cells showed swollen and desquamation with capillary rupture. This appearance was supported by the previous studies that found the leptospires in the lumen of blood vessels.

Moreover in all experimental organs, the red blood cells in both haemorrhagic area and lumen of blood vessels appeared positive golden brown stain. These cells might be covered or contacted with leptospiral antigens circulating in the blood stream. The cytoplasm of the neutrophils appeared also positive stain in both haemorrhagic area and lumen of blood vessels. These cells might have engulfed the leptospires and/or their antigens because the prime function of them was phagocytosis and digestion of the phagocytosed material, such as bacteria.

However, the leptospiral antigens in several areas of the infected organs which found in this study were presented by the positive golden brown coloration. The positive area corresponded to the pathological features of the infected organs observed in the previous studies. Therefore, the detected leptospiral antigens should be the primary cause of all lesions which occurred within the infected organs of the patients and the experimental animals associated with leptospirosis.

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APPENDIX

APPENDIX A
Bouin's solution

Bouin's solution (Bancroft *et al*., 1996)

1. Saturated aqueous picric acid solution	75 ml
2. 40% formaldehyde	25 ml
3. Glacial acetic acid	5 ml

Mix saturated aqueous picric acid solution, 40% formaldehyde and glacial acetic acid together. After mixing, it should be used immediately.

APPENDIX B

Tissue processing

1. Dehydration

This method was to replace the fixative and water in the tissue by dehydrating fluid, ethanol, as in the diagram 1.

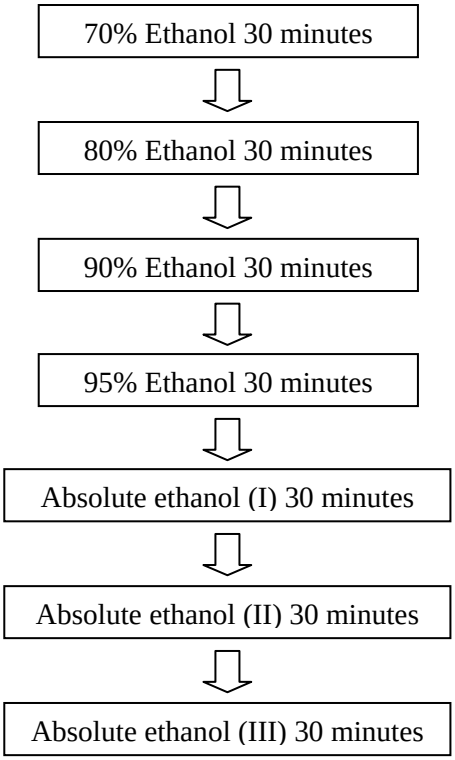

Diagram 1 Dehydration procedure.

2. Clearing

After dehydration, some dehydrating agents were left in the tissue block which was not miscible with the paraplast needed in the infiltration process followed. Therefore, the clearing agent, xylene, which was miscible in both dehydrating agent,

ethanol, and paraplast was needed in the clearing process. As a result, the xylene substituted the ethanol in the tissue block that eased the next step, infiltration. The tissue was immersed in the xylene. When the ethanol has been entirely replaced by most of the xylene, the tissue showed the translucent appearance (Diagram 2)

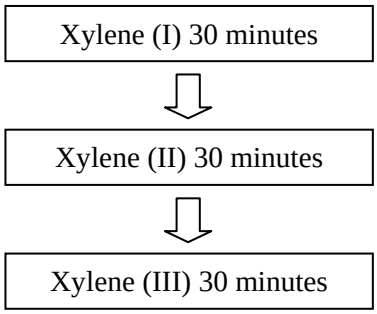

Diagram 2 Clearing procedure.

3. Infiltration

The infiltration process was done in order to replace the xylene by the embedding media, paraffin, in the tissue block. This step was done in the hot air oven at temperature between 56-60˚C (Diagram 3).

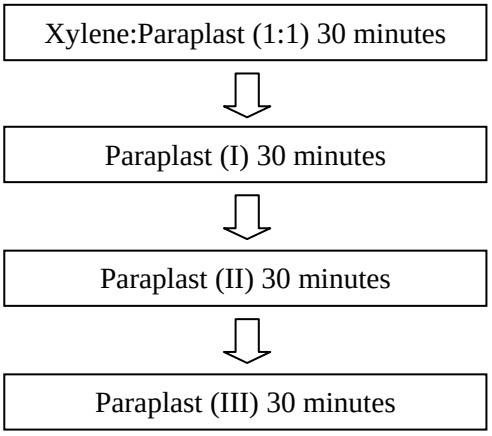

Diagram 3 Infiltration procedure.

4. Embedding tissue in the paraplast

The infiltrated tissue was placed in the melting paraplast occupied in the base of the mold and then immediately poured melting paraplast over the tissue until the mold was full. Let the mold cool on the ice plate. When the mold was cool, the paraplast had

solidified, the embedded tissue block could be removed from the mold. This tissue block was then ready for sectioning.

APPENDIX C
Procedure for the coating slide

Glass slides were dipped in the acetone for 10 seconds and followed by dipping in the 3% silane in acetone for 20 seconds and dipped in acetone 10 seconds and then these glass slides were washed by running tap water for 1 minute and dipped in distilled water 2 times. Finally, these glass slides were incubated in hot air oven at 56-60˚C overnight and kept in dry slide boxes. The process for coating the slides was show in diagram 4.

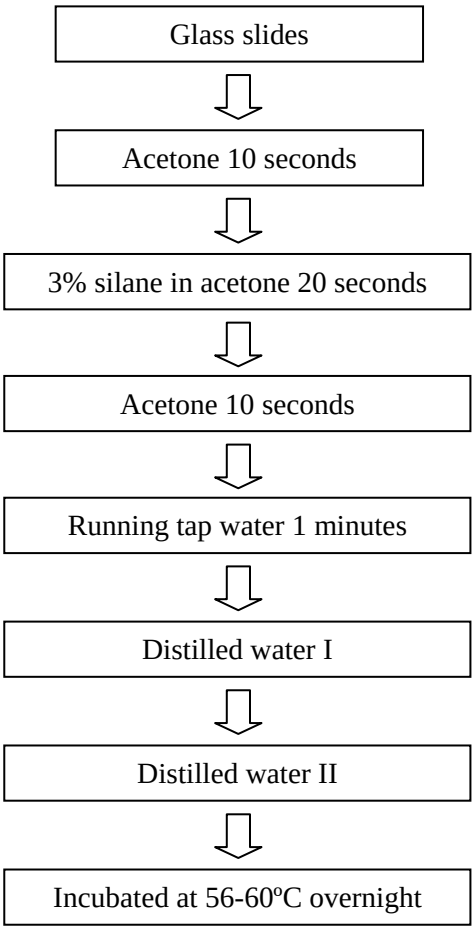

Diagram 4 Slide coating procedure.

APPENDIX D

Procedure of indirect immunoperoxidase staining technique

The sequence of this staining technique followed the rabbit ABC staining system kit protocol. First, deparafinization step by two steps of xylene for 3 minutes each, then rehydration by 2 times of absolute ethanol (3 minutes each), 2 times of 95% ethanol (3 minutes each) and 70% ethanol 3 minutes. Then, block the endogenous peroxidase by incubating in 3% H_2O_2 in PBS (Appendix E and F) 5 minutes for liver tissue and 7 minutes for lung and spleen tissue. After 3 minutes wash in deionized water and 2 times in PBS (5 minutes each), the sections were incubated in 1.5% blocking serum in PBS (Appendix G) at 37˚C in humidified chamber for 1 hour. Then the rabbit primary antibody specific for *L. interrogans* serovar pyrogenes was added at the dilution of 1: 1000 in PBS for 1 hour at 37˚C in humidified chamber, washed 3 times (5 minutes each) in PBS, incubated in biotinylated secondary goat anti-rabbit IgG 0.5 µg/ml (Appendix G) for 1 hour at 37˚C in humidified chamber, washed 3 times in PBS (5 minutes each), incubated in Avidin-Biotin enzyme reagent 1 µg/ml (Appendix G) for 30 minutes at 37˚C in humidified chamber, washed 3 times in PBS (5 minutes each), dropped peroxidase substrate (Appendix G) for 4 minutes and washed 5 minutes in distilled water. After washing, counter stained with Carazzi's haematoxylin (Appendix H) and washed with several times in distilled water and running tap water for 5 minutes for getting rid of the excess haematoxylin. Then, before mounting, slides were washed in distilled water, dehydrated by dipping in 2 times of 95% ethanol (3 minutes each), 2 times of absolute ethanol (3 minutes each) and 2 times of xylene (3 minutes each) (Diagram 5).

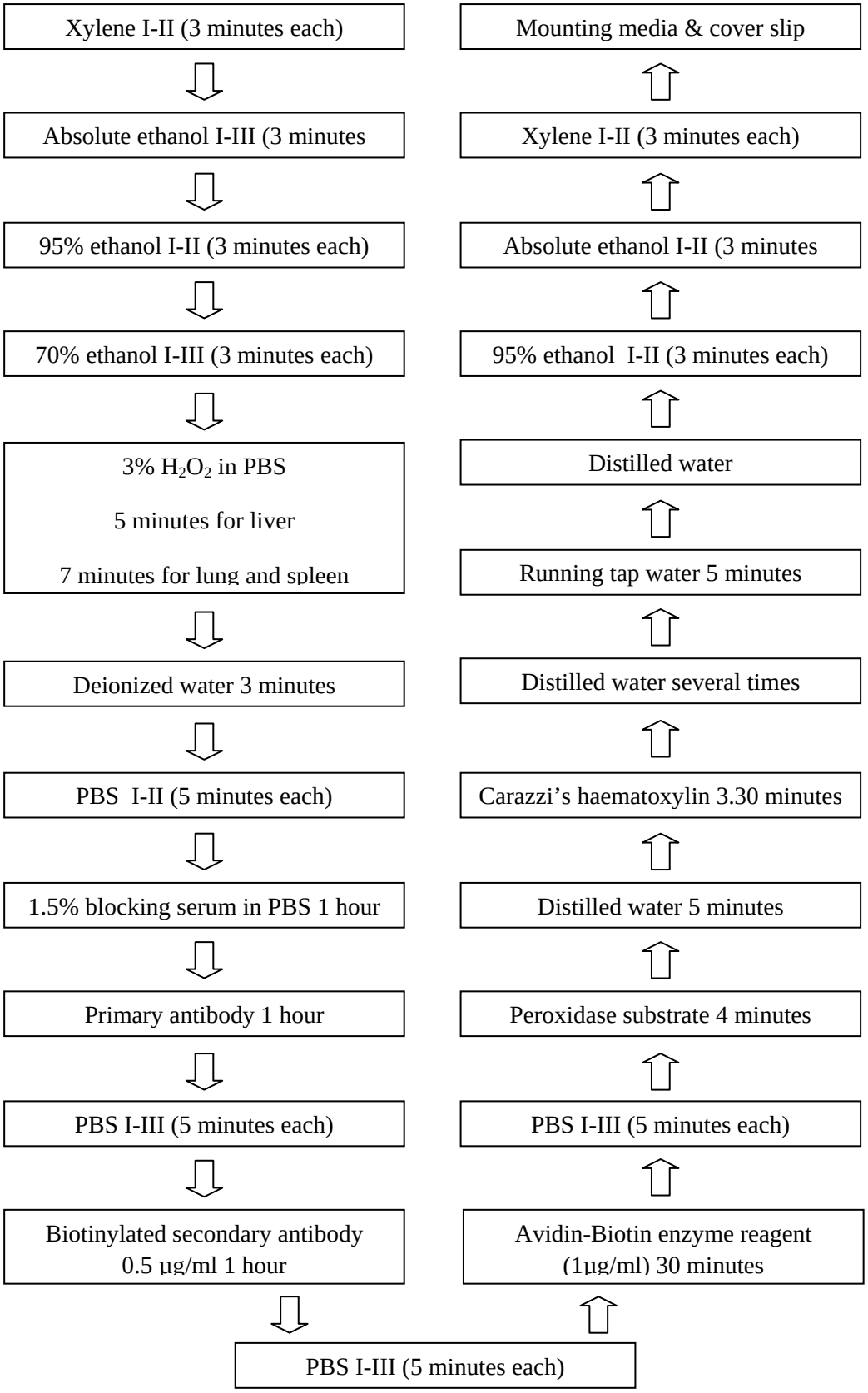

Diagram 5 Indirect immunoperoxidase staining procedure.

APPENDIX E

Phosphate buffer saline (PBS)

Phosphate buffer saline, PBS (Bancroft *et al*., 1996)

Stock solution

Stock A: sodium dihydrogen orthophosphate	3.12 g
distilled water	100.00 ml
Stock B: disodium hydrogen orthophosphate	2.83 g
distilled water	100.00 ml

Working solution, pH 7.20

Mix 14.0 ml of stock A with 36.0 ml of stock B and made up to 100 ml with distilled water. The pH of this working solution was checked by pH meter and adjusted by sodium dihydrogen orthophosphate (acid) and disodium hydrogen orthophosphate (base).

APPENDIX F

H_2O_2 3% in PBS

H_2O_2 3% in PBS

H_2O_2	3 ml
PBS	97 ml

Mix H_2O_2 and PBS in the coplin jar. After preparation, it should be used immediately.

APPENDIX G

Preparation of ABC staining system working solutions

1. Blocking serum

Normal blocking serum stock	75 µl
PBS	5 ml

Mix normal blocking serum with PBS in the bottle 1 (blue cap).

2. Biotinylated secondary antibody

Normal blocking serum stock	75 µl
Biotinylated secondary antibody stock	25 µl
PBS	5 ml

Mix normal blocking serum stock, biotinylated secondary antibody stock and PBS in the bottle 2 (green cap).

3. AB enzyme reagent

Avidin	50 µl
Biotinylated HRP	50 µl
PBS	2.5 ml

Mix Avidin, biotinylated HRP and PBS in the bottle 3 (purple cap). Let the mixed solution stand for approximately 30 minutes before use.

4. Peroxidaes substrate

Distilled water	1.6 ml
Substrate buffer 10x concentrate	5 drops
DAB chromogen 50x concentrate	1 drop
Peroxidase substrate 50x concentrate	1 drop

Mix Distilled water, substrate buffer, DAB chromogen 50x concentrate and peroxidase substrate in the bottle 4 (yellow cap).

After this working solution of ABC staining system was used, it should be kept at 2-8 ˚C, do not freeze.

APPENDIX H
Carazzi's haematoxylin

Carazzi's haematoxylin (Bancroft *et al*., 1996)

Haematoxylin	50.0 g
Glycerol	100.0 ml
Potassium alum	25.0 g
Distilled water	400.0 ml
Potassium iodate	0.1 g

The preparation of Carazzi's haematoxylin was adapted from Bancroft *et al*., 1996. The haematoxylin was dissolved in the glycerol (called haematoxylin solution), and the potassium alum was dissolved in distilled water (called alum solution). The alum solution was added slowly to the haematoxylin solution, mixing very well after each addition. Then added the potassium iodate in the mixing solution and mixed at gentle warming on the heater.

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